A STUDY OF THE EPIDEMIOLOGY AND PATHOGENICITY OF *SALMONELLA VIRCHOW*

Andrea Michelle Sullivan B. Sc. (Hons)

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DECLARATION

The work presented in this thesis is, to the best of the author's knowledge and belief, original, except as acknowledged within the text. It has not been submitted previously, either in full or in part, for another degree at this or any other institution.

Andrea Sullivan

A.M. Sullivan.

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I dedicate this thesis to my loving parents, Lillian and Kevin and to my darling fiancé, Craig.

SUMMARY

Salmonella virchow is a seldom studied serovar of *Salmonella* despite it being in the top three serovars isolated from humans in Australia for the last six years. It is also prevalent in the United Kingdom and from 1981-1986 the number of human infections increased by 30% (Humphrey *et al.*, 1988). There were two broad aims of this thesis. The first was to undertake an epidemiological study of *S. virchow* in Australia. The second was to investigate the pathogenicity of *S. virchow* focussing on two potential virulence factors, namely SEF17 fimbriae and lipopolysaccharide (LPS).

The epidemiological investigation began with an evaluation of five typing methods, applied to ninety-five *S. virchow* isolates from varied sources including poultry, other animals, human infections and sewage effluent. The five typing methods used were antimicrobial susceptibility testing, phage typing, plasmid profiling, ribotyping and IS*200* typing. An index of discrimination (D) was calculated for each of the typing methods. The discriminatory index ranges from 1 which is the most discriminatory to 0 which is the least discriminatory. Antimicrobial susceptibility proved to be the most discriminatory (D = 0.593), closely followed by phage typing (D = 0.530). The molecular methods such as plasmid profiling (D = 0.360), ribotyping (D = 0.359) and IS*200* typing (D = 0.211) were less discriminatory.

Plasmid profiling and ribotyping were applied in an epidemiological investigation of nine *S. virchow* isolates obtained from an outbreak. The State Health Department of Queensland provided the isolates which had been obtained from faecal samples of individuals involved in a food-borne outbreak. Ribotyping was applied because it had shown strong correlation with the epidemiological data when applied to the large group of *S. virchow* isolates. It was important to determine if this method would be as discriminatory with a group of known related isolates. Phage typing could not be used because the isolates only became available late in the project and there was not sufficient time for the Public Health Laboratory Service, London to receive the isolates and determine the results. Plasmid profiling and

ribotyping both found the nine strains to be indistinguishable from each other. No isolates from the food suspected of being the source of the outbreak were available to be typed.

The results of the five typing methods were combined and this resulted in a discriminatory index of 0.95 and the formation of 30 subgroups from the ninety-five isolates. Four of these subgroups represented clonal lines of *S. virchow* because the members of these groups were indistinguishable by all five methods. Other groups of highly related isolates also existed, the members of which differed by only one or two typing methods or because not all of the methods had been applied. Importantly the largest clonal line contained four human and nine poultry isolates and confirmed the hypothesis that poultry is a reservoir of *S. virchow* for human infection and is also a vehicle of transmission.

The second area of investigation in this thesis was the pathogenicity of *S. virchow*. The approach taken was to study two potential virulence factors in isolation and establish if either could explain this serovars ability to cause extra-intestinal infections.

This thesis reports, for the first time, the presence of SEF17 on *S. virchow*. SEF17 are thin, aggregative fimbriae found on *S. enteritidis* which are responsible for fibronectin and collagen binding. Four phenotypic tests were employed to screen the ninety-five *S. virchow* isolates for the likelihood of fimbriae. The four phenotypic tests were: the binding of Congo Red, growth as a pellicle in CFA broth cultures and colony morphology on colonization factor antigen (CFA) agar and T medium. Seventy-nine of the ninety-five isolates (83%) indicated the presence of SEF17 by one or more of these tests. These phenotypic tests relied on the presence of fimbriae whose expression is variable and therefore all ninety-five isolates were also tested for the gene encoding SEF17, *agfA,* using PCR. The gene was detected in ninety-two of the ninety-five isolates (96.8%). The nucleotide sequence of the 258bp PCR product was determined for one of the isolates and after translation found to be identical to the amino acid sequence of the SEF17 from *S. enteritidis*. Electron microscopy and negative staining allowed the fimbriae-like structures on the surface of another of the *S. virchow* isolates to be visualized. In addition, thirty-four of the

forty-seven isolates (72%) tested by immunoblotting were positive for a protein of ca. 17kDa, which was immunoreactive to the SEF17 specific antiserum.

Lipopolysaccharide (LPS) was also investigated as a possible virulence factor. All of the ninety-five isolates were analysed for their LPS structure. All demonstrated wild type (smooth) LPS however, three types were distinguished by subtle differences in the electrophoretic patterns. These differences were due to variations in the O-side chain of the isolates and the three types were designated type A, B and C. The most prevalent type (63%) was the true wild type LPS and was designated type B. Type A was very similar and was observed for a further 21 isolates. Type A had a shorter laddered appearance than type B due to the repeating sugar units in the O-specific side chain. This indicated the presence of a smaller number of sugar units in the side chain of these isolates. All of the remaining isolates formed type C which had the unique feature of a core oligosaccharide band of less relative mobility than either type A or B, as determined by polyacrylamide gel electrophoresis. This decreased mobility indicated that the core oligosaccharide of this LPS was larger than the core oligosaccharides of type A and B LPS. In addition, type C produced a laddered electrophoretic pattern of doublet and triplet bands. The presence of doublets and triplets suggested that the LPS samples from these isolates were comprised of more than one LPS molecule, indicated by the presence of mixed LPS types. More than one pathway of LPS biosynthesis and assembly in these isolates has been proposed in this thesis as a likely explanation.

When repeated testing was performed it was observed that the LPS type of the isolates changed and all isolates demonstrated type B LPS. Several hypothesis were examined to explain this result including an investigation of the effect of incubation time and the effect of subculture on LPS expression. No definitive explanation was determined but evidence would suggest that the LPS expressed by an isolate changes in response to a change in the organisms environment.

In addition to the epidemiological and pathogenicity studies, a cell culture assay was also evaluated as a model for invasiveness. Isolates were assigned as either invasive or non-invasive based on their site of isolation. Isolates from blood, urine and liver were deemed invasive while isolates from faeces, animal feed

components and sewage effluent were considered non-invasive. Nine invasive and eleven non-invasive isolates were tested using the invasion assay to determine the percentage of bacterial cells inoculated onto the HEp-2 cell monolayer which were able to invade the cell cultured cells. No significant difference between the level of invasiveness between the invasive and non-invasive groups was observed. The average percentage of invasion was extremely low for the invasive and non-invasive groups at 0.151% and 0.148%, respectively. Nevertheless, the cell culture assay was able to show a significant difference between the invasion rate of the control strain, *S. typhimurium,* and the *S. virchow* isolates. This result indicated that the assay had potential as an invasion assay but the arbitrary designation of the *S. virchow* isolates as invasive and non-invasive based on their site of isolation was inaccurate and inappropriate for the evaluation of these isolates.

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ABBREVIATIONS

%	percent
α	alpha
β	beta
μ	micro
Ω	ohms
1492r	pUC M13 1492 reverse primer
27f	pUC M13 27 forward primer
A	adenine
A_{260nm}	absorbance at 260nm
ANGIS	Australian National Genome Information Service
ANOVA	analysis of variance
APS	ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BG	brilliant green agar
bisacrylamide	N,N,1-methylene bisacrylamide
BLAST	Basic Local Alignment Search Tool
bp	basepairs
BS	bismuth sulphite agar
BSA	bovine serum albumin
C	cytosine
ca.	approximately
CAT	chloramphenicol acetyltransferase
CFA	colonization factor antigen
cfu/ml	colony forming units per millilitre
CR	congo red
D	discrimination index
Da	dalton
dATP	deoxy adenosine triphosphate
ddNTP	dideoxy nucleotide triphosphate
dH_2O	distilled water
DIG	digoxygenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
E. coli	*Escherischia coli*
EDTA	ethylenediamine tetra-acetic acid
EIRDC	Egg Industry and Research Council
EM	electron microscopy
EMBL	European Molecular Biology Laboratory
FBS	foetal bovine serum
g	gram
G	guanine
HCl	hydrochloric acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hr	hour
IMVS	Institute of Medical and Veterinary Research
IPTG	isopropyl-β-D-thiogalactopyranoside

IS*200*	insertion sequence 200
isol.	isolate
IST	IS*200* type
kb	kilobase
kDa	kilodalton
KH_2PO_4	potassium dihydrogen orthophosphate
L	litre
LB	Luria-Bertani
LB_{amp100}	100mg ampicillin per litre of LB medium
LMG	Lysine-Mannitol-Glycerol agar
LMP	low melting point
LPS	lipopolysaccharide
m	metre
M	molar
mA	milliamps
MDU	Medical Diagnostic Unit, University of Melbourne
MEE	multilocus enzyme electrophoresis
mg	milligram
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
Na_2HPO_4	di-sodium hydrogen orthophosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
O/N	overnight
°C	degrees Celsius
ORFs	open reading frames
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG_{8000}	polyethylene glycol, average molecular weight 8000
PFGE	Pulsed Field Gel Electrophoresis
PHLS	Public Health Laboratory Service
PT	Phage type
QDPI	Queensland Department of Primary Industry
RAPD	Random Amplification of Polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
REA	Restriction Enzyme Analysis
RFLP	Restriction Fragment Length Polymorphism
RILT	rabbit ileal loop test
RILT	rabbit ileal loop test
RMIT	Royal Melbourne Institute of Technology
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
RT	ribotype

RV	Rappaport-Vassiliadis broth
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
SE	*Salmonella enteritidis*
sec	second
sp.	species
ss	single stranded
SSC	standard saline citrate
T	thymine
t-PA	tissue-type plasminogen activator
TAE	Tris/acetic acid/EDTA buffer
Taq	*Thermus aquaticus*
TBE	Tris/boric acid/EDTA buffer
TCR	T medium containing Congo red
TE	Tris/EDTA buffer
TEMED	N,N,N^1,N^1-tetramethylethylenediamine
T_M	melting temperature
Tris-HCl	tris (hydroxymethyl) aminomenthane hydrochloride
TSA	Tryptone soya agar
U	uracil
UK	United Kingdom
UV	ultraviolet
V	Volts
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
XLD	Xylose-lysine-deoxycholate agar

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CHAPTER 1

LITERATURE REVIEW

Chapter 1 : Literature Review

This literature review presents the knowledge that exists in the literature which relates to the experimental sections of this thesis. It does not attempt to provide an exhaustive review of the depth of understanding of *Salmonella* spp.

1.1 Salmonellae and S. virchow

1.1. a) Morphology, taxonomy and nomenclature

Salmonella, a genus of the family *Enterobacteriaceae,* comprises Gram-negative rod - shaped bacteria which do not produce endospores and are usually motile (Holt *et al.*, 1984). The taxonomy of *Salmonella* is complex and has not been fully resolved. There are no guidelines, no standard system of classification and no universally accepted type cultures at this time (Holt *et al.*, 1994). The use of biochemical, cultural and antigenic features as well as DNA relatedness studies have shown that the *Salmonella* genus consists of one species with seven subgroups or subspecies (Le Minor & Popoff, 1987; Jay & Davey, 1989) designated *cholerasuis*, *salamae*, *arizonae*, *diarizonae*, *houtenae, bongori* and *indica* (Le Minor & Popoff, 1987). It has also been suggested that the species name *cholerasuis* be replaced by the name *enterica* because *cholerasuis* is already used as a serovar name (Le Minor & Popoff, 1987).

There has also been a proposal that there should be two species of *Salmonella* due to the elevation of subspecies *bongori* to species level (Reeves *et al.*, 1989) This proposal was based on results of multilocus enzyme electrophoresis studies which showed that the subgroup V, or subspecies *bongori* as it is now known, met the criterion set by Selander *et al.* (1985), for the genetic distance required to be a separate species.

The seven subspecies are further divided into over 2000 serovars (or serotypes) based on their antigenic structure of O and H antigens. The O-antigen also known as the somatic antigen, is associated with the lipopolysaccharide structure of bacterial cells and can be used to group *Salmonella* strains into O groups (Holt *et al.*, 1994).

These O groups contain all strains of *Salmonella* which express the same somatic antigens. The H antigens are flagella antigens and the presence of different flagellar antigens allows the strains to be further differentiated into serovars. *S. virchow* belongs to the species *enterica* subsp. *cholerasuis* serovar *virchow*. Serovar *virchow* has the antigenic formula 6,7:r:1,2 which represents the O-antigens: phase 1 H antigen: phase 2 H antigens, respectively. The correct nomenclature for *Salmonella* is to write the genus, species, subspecies and serovar as shown above without including the O group. However the abbreviated nomenclature of genus followed by serovar can also be used and has been adopted herein.

1.1. b) Pathogenicity and epidemiology

Salmonellosis is an important public health consideration the world over with estimates of the number of infections in humans being between 800 000 and 3 700 000 annually in the United States of America alone (Chalker & Blaser, 1988). It is generally acknowledged that a large number of cases are due to food-borne infections which cause gastroenteritis. However even infections such as gastroenteritis can be serious, and indeed life threatening in immuno-compromised hosts (Chalker & Blaser, 1988). *Salmonella* serovars generally belong to one of three groups :

- adapted to man - for example *Salmonella typhi*, *Salmonella paratyphi* A and B;
- adapted to animals - for example *Salmonella pullorum* and *Salmonella cholerasuis;* or
- unadapted - includes serovars which infect man and animals and represents the majority of serovars.

The infections caused by these three groups can be quite different. Infection with a member of the group adapted to man is characterized by a low infectious dose, an incubation period of 10-20 days, bloodstream invasion and fever. This infection is generally acquired by ingestion of contaminated food or water. Infection in humans with an animal adapted serovar can result in gastroenteritis and some serovars, such as

Salmonella cholerasuis, have been reported to cause bloodstream invasion. The largest group of *Salmonella* contains serovars which infect both humans and animals and these most commonly cause gastroenteritis, with a short incubation period. Some of these serovars are also capable of causing septicaemia and extra-intestinal infections including localized infections in tissues associated with the gastrointestinal tract or other tissues such as bone and heart (Grau, 1989).

S. virchow is a member of the unadapted group and is most commonly associated with a mild self-limiting gastroenteritis-type illness. Recent reports indicate that this serovar is an increasing cause of invasive salmonellosis including septicaemic illness (Taha & Peden, 1987; Ashdown & Ryan, 1990), localized infections such as bone infection (osteomyelitis) (Ingram & Redding, 1988), heart tissue involvement (Echevarria *et al.*, 1989) and pathology at other extra-intestinal sites (Wilkins & Roberts, 1988; Sechter *et al.*, 1991).

S. virchow is a prevalent serovar both in Australia and overseas. It has been in the top three serovars isolated from humans in Australia every year from 1988 until 1995 except 1991, when it was sixth. Between 1988 and 1995 the incidence of *S. virchow* as a percentage of the total human clinical *Salmonella* isolations fluctuated between 4.1 and 7.4% (National Salmonella Surveillance Scheme, 1988, 1989, 1990, 1991, 1992, 1993a, 1994a, 1995a). Similarly, in the United Kingdom, *S. virchow* is in the top three serovars isolated from humans, after *S. enteritidis* and *S. typhimurium*. *S. virchow* isolations accounted for 7.9% of the total *Salmonella* isolations in the UK, during the period of 1981-1985 (Barrell, 1987).

Salmonella spp. as food-borne pathogens are associated with various foods, food animals and environmental sites. During the period 1993-1995 in Australia, *S. virchow* was isolated not only from humans but from chickens, ducks, cattle and a dairy factory environment (National Salmonella Surveillance Scheme, 1993b, 1994b, 1995b). The isolation of *S. virchow* from animals is also well documented overseas and in particular in England, Wales, Scotland and the Netherlands (Humphrey *et al.*, 1988; Reilly *et al.*, 1988; Giessen *et al.*, 1991). All of these reports implicate poultry as a major vehicle of transmission of *Salmonella* to humans. Not only is *Salmonella* recognized but *S. virchow* in particular is reported in all cases to be prevalent in poultry. Humphrey *et al.* (1988) reported that poultry associated *S. virchow* outbreaks

were common in the early 1970s but had declined since. However in the period 1981-86 there was a 30% increase in human isolations and poultry was again implicated as the source. Reilly *et al.* (1988) reported that poultry meat accounted for over 50% of outbreaks where a food vehicle was identified and of the 321 *Salmonella* serovars that were isolated in that investigation, *S. virchow* was the second most common serovar from both poultry meat associated outbreaks and poultry meat in general and the third most common serovar isolated from humans. A study from the Netherlands (Giessen *et al.*, 1991) reported isolation of *S. virchow* from 31% of poultry flocks and found it to be the most prevalent serovar, after *S. infantis*.

A strong relationship between poultry and *S. virchow* in Australia has not been investigated despite the same incidence of *S. virchow* in the human population in Australia as reported overseas. An essential tool in such an investigation would be a typing method which would determine if *S. virchow* isolates from humans and poultry in Australia are related.

Post-process contamination or consumption of under prepared foods is the most common route of *Salmonella* infection. For this reason food handling practices can have a large influence on the number of infections. By way of example, until 1983 milk was the most important food vehicle causing *Salmonella* outbreaks in Scotland. However, in 1983 compulsory heat treatment (pasteurization) of cows' milk was introduced and the incidence of milk related salmonellosis decreased and poultry meat became the most important food source of salmonellosis (Reilly *et al.*, 1988). Barrell (1987) also demonstrated the effect of food preparation by showing the different isolation rates of *Salmonella* from cooked and raw product. *Salmonella* was found in ≤0.5% of most cooked meats compared with isolations from raw meats of 28% for poultry, 18% for lamb and 12% for sausage meat.

Another important consideration of raw products relates to eggs. Intact shell eggs were identified as the major vehicle of transmission of *S. enteritidis* in Britain when its incidence increased markedly in the early 1980s (Cox, 1995). This was because transovarian transmission from an infected bird to the egg occurs before the shell is in place. Consequently, products made with raw eggs such as home-made mayonnaise have been shown to be vehicles of transmission and *S. enteritidis* has even been demonstrated to survive cooking of whole eggs when the yolk remains liquid (Cox, 1995).

1.2. Epidemiological Typing Methods of Salmonella

Generally, typing refers to the application of various methods to subdivide a group of isolates. As explained earlier, all members of the genus *Salmonella* belong to one species, *S. enterica* (Le Minor & Popoff, 1987). Within the species, there are seven subspecies based on biochemical properties. Strains of *Salmonella* are further subdivided into serovars based on antigenic structure, that is the somatic (O) and flagellar (H) antigens. Therefore by employing typing methods to *Salmonella* further subdivisions within a serovar can be made. Typing methods are commonly used in outbreak situations to aid in identifying the source of an outbreak and identifying individuals infected from the source. Several of the most common typing methods are discussed below and these represent both phenotypic and genotypic techniques.

1.2. a) Phage typing

Phage typing differentiates bacterial strains based on their sensitivity to bacteriophages (phages). The bacterial strain being typed is exposed to a panel of phages and the sensitivity of the strain to the different phages generates a pattern which generally conforms to a phage type. Phage typing began in 1931 when specific phages were used for the identification of *Shigella* strains (Ackermann & DuBow, 1987).

Phage typing can be performed using either phages isolated from the local environment or using a standard set of phages. However, if typing is performed using phages isolated locally comparisons of the results to those in the literature is difficult because different phages were used. This was highlighted by the study of Milch *et al.* (1985a) in which phage typing of *S. typhimurium* was reported and the results from Hungary could not be compared to reports from other countries because different phages had been used. Therefore many of the clinically important pathogens have internationally agreed sets of phages and a standard scheme for reporting results. When phage typing is performed with standard schemes the results from different areas can be directly compared. There are schemes for many *Salmonella* serovars including

S. typhi, *S. paratyphi B*, *S. enteritidis* and *S. virchow* (Ackermann & DuBow, 1987; Olsen *et al.*, 1993).

A genuine limitation of phage typing is the inability to obtain epidemiologically significant results when one phage type is predominant in a geographic location. This was demonstrated recently when a world-wide increase in the incidence of *S. enteritidis* phage type 4 occurred. Phage type 4 became the predominant phage type in many countries and therefore identification of this phage type did not provide definitive information about the origin of that strain (Olsen *et al.*, 1993).

It should also be noted that conversion of phage types can occur and preparation and propagation of phages must be carried out carefully. Phages should routinely be checked by electron microscopy to ensure they are maintained and are not being replaced by other bacterial viruses (Ackermann & DuBow, 1987).

A further limitation of phage typing is that some strains of bacteria will not be able to be phage typed. This occurs when strains are susceptible to phages but the pattern of sensitivity has not been reported previously. This is referred to as reacts but does not conform (RDNC).

The current standard scheme for phage typing of *S. virchow* was established by the *Salmonella* Reference Laboratory, Public Health Laboratory Service (PHLS), Colindale, London (Chambers *et al.*, 1987). The scheme consists of 13 phages from two sources - six phages were isolated as lysogenic strains of *S. virchow* and seven were from the typing scheme of another *Salmonella* serotype, *S. thompson*, which shares somatic antigens with *S. virchow* (O antigens 6,7). By 1993, 57 phage types of *S. virchow* had been identified using the *S. virchow* scheme (Torre *et al.*, 1993). However, large proportions of the isolates belonged to a small number of phage types which meant that phage typing alone was not highly discriminatory for *S. virchow*. Therefore, to obtain epidemiological data it became necessary to combine phage typing with other typing methods.

1.2. b) Biotyping

Biotyping is the grouping of isolates based on their reaction in biochemical tests such as fermentation of substrates, motility tests and tests for the expression of heamagglutinating fimbriae (Threlfall & Frost, 1990). There are schemes for *S. agona* (Barker & Old, 1982), *S. gallinarum / S. pullorum* (Christensen *et al.*, 1992), *S. havana* (Katouli *et al.*, 1992) , *S. montevideo* (Old *et al.*, 1985) and several other serovars (Olsen *et al.*, 1993) and the scheme for *S. typhimurium* has been developed into a two tier scheme (Duguid *et al.*, 1975). There is no biotyping scheme in the literature for *S. virchow*.

Biotyping is reasonably laborious and there seems to be a difference in opinion as to its discriminatory value, depending on which serotype is being typed (Olsen *et al.*, 1993). Like phage typing, biotyping probably has the most to offer if used in conjunction with one or more other typing method.

1.2. c) Antibiotic susceptibility testing

Antibiotics are low molecular weight microbial metabolites, which at low concentrations inhibit the growth of other microorganisms (Lancini *et al.*, 1995). Antibiotics are strictly defined as natural products of microorganisms but may be chemically modified or artificially synthesized. There are approximately 10 000 known and chemically defined antibiotics and these can be divided broadly by their spectrum of activity. Antibiotics are classed as antiviral, antibacterial, antifungal, and antiprotozoal depending on the microorganisms which are sensitive (Lancini *et al.*, 1995).

It is the structure of bacterial cell wall which plays the major role in determining the sensitivity of bacteria to different antibiotics. The structure of the Gram-positive cell wall is considerably more permeable and therefore many more antibiotics are able to penetrate these cells. Antibiotics that are able to penetrate the

more impermeable Gram-negative wall will most likely have activity against both Gram-positive and Gram-negative cells.

Antibiotic susceptibility testing is the determination of the level of sensitivity microorganisms have to a range of antibiotics *in vitro*. Antibiotic susceptibility testing can be used to direct the rational and optimal prescription of antibiotics in the treatment of clinical infections. In epidemiological studies, the antimicrobial susceptibility and resistance profile of a strain serve as fingerprints. By comparing the profiles, closely related isolates such as those involved in a nosocomial infection or food-borne outbreak, can be identified.

Appendix 1 contains an overview of the major groups of antimicrobial agents and some of the antibiotics from those groups which can be used in susceptibility testing and their mechanisms of action and resistance.

The increase in incidence in some parts of the world of various serovars such as *Salmonella enteritidis*, has caused an increase in the need for methods able to subtype serovars. Antibiotic susceptibility testing has been adopted for this purpose (Bezanson *et al.*, 1985; Milch *et al.*, 1985b; Rampling *et al.*, 1990; Borrego *et al.*, 1992; Stubbs *et al.*, 1994; Nair *et al.*, 1995; Cox *et al.*, 1996a). Susceptibility to antimicrobial agents can be tested by several methods including agar diffusion and both agar and broth dilution tests. Susceptibility testing is well established and performed routinely in clinical labs.

There are, however, two major limitations to susceptibility testing as a typing tool. The first is that the results obtained are only an indication of the organism's susceptibility *in vivo* due to the pharmokinetics of the drug itself and many variables of the test conditions which can influence the result. Some of these variables include the inoculation size, incubation conditions and the media used (Ericsson & Sherris, 1971). Secondly, resistance to many antimicrobial agents can be encoded on plasmid DNA. Plasmid DNA encoding resistance genes enables the resistance to move between certain strains relatively rapidly (Poppe *et al.*, 1995). This transfer of resistance is a contributing factor to the need for antibiotic susceptibility testing to be performed in combination with other typing methods (Borrego *et al.*, 1992).

Similarities in susceptibility profiles between isolates of human origin and animal or environmental origin can be used as an indicator of a possible source of

clinical infection (Nair *et al.*, 1995). It has been suggested that the prevalence of the same resistances in animals and humans is due to the selective pressure exerted on the bacterial population when the same antibiotic agents are used in both human and veterinary medicine. The practice of using antimicrobial agents from the same class of active compounds to treat illness in humans and as growth stimulants or prophylactic agents in medicated veterinary feeds to food animals may favour the establishment of a population of resistant bacteria. These resistant bacteria can subsequently be excreted by animals, contaminating the carcasses and causing infections in humans by the ingestion of under-prepared foodstuffs (Poppe *et al.*, 1995).

The majority of studies into *S. virchow* antibiotic susceptibility have focused on the incidence of antibiotic resistance and not utilised the method for typing of isolates (Threlfall *et al.*, 1992, 1993; Frost *et al.*, 1996; Ramos *et al.*, 1996). *S. virchow* is recognized as capable of causing invasive infections in humans and so possession of resistance to antibiotics has significance in determining effective treatments (Frost *et al.*, 1996).

The only thorough antibiotic resistance investigation of *S. virchow* was performed by Threlfall *et al.* (1993), who reported the resistance of *S. virchow* isolated from humans and poultry to individual antimicrobial agents. Threlfall *et al.* (1993) found that the incidence of furazolidone resistance in *S. virchow* isolates from poultry in the UK in 1990 was 24% and this high incidence was linked to the routine use of nitrofurans such as furazolidone in the British poultry industry at the time. The study also reported the incidence of furazolidone resistance in human clinical isolates and showed that it had increased from 9% in 1981 to 71% in 1990. In contrast, the increase of multiply resistant strains of *S. virchow* for the same period was only from 1% to 11%. This circumstantial link between furazolidone resistance in poultry and human strains of *S. virchow* supported the hypothesis that poultry are the main reservoir of *S. virchow* for humans.

1.2. d) Plasmid profiling

Plasmid profiling is the investigation of strains for the number and size of plasmids present. Plasmids are circular forms of DNA which replicate in bacterial cells independent of genomic DNA replication. In addition to encoding genes for their own replication, these molecules of DNA can encode various other genes including virulence genes, antibiotic resistance genes and heavy metal resistance genes. Bacterial cells can contain more than one plasmid at a time and these can be of different sizes. When a bacterial cell divides, a copy of the plasmid DNA is distributed to each of the daughter cells.

Plasmid profiling is a genotypic typing method distinct from antibiotic susceptibility and serotyping because it involves the characterization of DNA. Genotypic methods, on the whole, are considered to be more reliable than phenotypic typing methods because they do not require the expression of genes to produce a phenotype (Olsen *et al.*, 1993).

Plasmid profiling should not be confused with plasmid fingerprinting. Plasmid fingerprinting involves the differentiation of plasmid DNA by restriction enzyme digestion and comparison of the restriction patterns generated (Threlfall & Frost, 1990). Plasmids which appear to be the same based on plasmid profiling may be different at the nucleotide sequence level (Olsen *et al.*, 1993) and this can be determined by the use of fingerprinting.

There are some features of plasmid DNA which limit its potential as a typing method. The first limitation is that plasmid DNA can exist in several conformational forms, covalently, closed circular (ccc), open circular (oc) and linear molecules. Therefore what may at first appear to be two or three plasmids in one strain could be several forms of one plasmid depending on which conformational forms dominate.

Plasmid DNA can be transferred between bacteria by conjugation or by mobilization of other plasmids present in the host. This transfer of plasmid DNA makes the use of plasmid presence and absence as a typing method problematic.

Olsen *et al.* (1994a) investigated the stability of plasmids in *Salmonella* stored at different temperatures over a period of two and a half years. Storage at -80°C in

glycerol resulted in no observed loss of plasmids and storage at refrigerator temperatures in sugar free media resulted in only small losses. However, storage of stab cultures at room temperature or above resulted in loss of plasmids, with the frequency of loss increasing with the time of storage.

Plasmid profiling has been applied to several serovars of *Salmonella* with limited success (Whiley *et al.*, 1988; Harrington *et al.*, 1991; Borrego *et al.*, 1992; Morris *et al.*, 1992). A study in which 13 serovars of *Salmonella* were isolated from food, epidemic outbreaks, the environment and sporadic clinical cases, concluded that plasmid profiling was unable to identify epidemiologically linked isolates (Borrego *et al.*, 1992). Also in a study of *Salmonella sofia* from Australia and Israel, Harrington *et al.* (1991) concluded that plasmid profiling was not sufficiently discriminatory. Several patterns were observed but most were common to all groups of isolates, both chicken and human.

However, other studies have found plasmid fingerprinting to be more discriminatory for subtyping. Bezanson *et al.* (1985) used plasmid profiling of *S. typhimurium* phage type 10 isolates to confirm the source of an outbreak. The plasmid profiles of isolates from humans and the suspected cheese were identical. However, upon plasmid fingerprinting analysis, the plasmids were found to be similar but not identical indicating distinct plasmids and therefore different strains. This suggested that the illness had been caused by double infection, a conclusion which had not been demonstrated by other traditional typing methods.

Two studies have used plasmid profiling to type *S. virchow* strains (Rivera *et al.*, 1991; Torre *et al.*, 1993). In the study of Torre *et al.* (1993), 40 isolates from clinical infections and food animals were investigated for the presence of plasmid DNA. Of the 40 isolates, 32 had one or more plasmids ranging in size from 2 - 140 MDa. The study also included investigation of ten unrelated isolates of *S. virchow* phage type 31; half of them contained plasmid DNA and two patterns were observed. This indicated that plasmid profiling has the potential to provide further differentiation within groups of *S. virchow* strains.

1.2. e) Genomic DNA typing methods

A range of typing methods exist which differentiate strains based on the analysis of the genomic DNA. For the purpose of this review these methods have been grouped broadly into three types :

- restriction analysis of the total genomic DNA;
- hybridization of the digested genomic DNA and probing either with random fragments as probes or probes designed to target specific sequences; and
- PCR-based methods which employ amplification of either random fragments of the genomic DNA or specific sequences.

1.2. e (i) Restriction enzyme analysis and Pulsed field gel electrophoresis

Restriction enzyme analysis (REA) is the generation of a restriction pattern by the restriction enzyme digestion of genomic DNA. This analysis is based on the assumption that strains from the same origin/location will have the same genome and identical restriction enzyme sites which will result in the same restriction pattern (Olsen *et al.*, 1993). The difficulty in applying this method is that the profiles generated from genomic DNA are complex and interpretation is limited by the large number of bands present. Pulsed field gel electrophoresis (PFGE) is a method employed to improve the resolution of the numerous restriction fragments generated by REA.

REA and PFGE have often been applied to the investigation of outbreaks involving *Salmonella* serovars including *S. agona*, *S. gallinarum*, *S. typhi*, *S. enteritidis* and *S. infantis* (Christensen *et al.*, 1994; Murase *et al.*, 1996; Navarro *et al.*, 1996; Threlfall *et al.*, 1996; Wegener & Baggesen, 1996).

Wegener and Baggesen (1996) used REA followed by PFGE to show that an outbreak *of S. infantis* responsible for over 500 human clinical cases originated from a pig herd supplied to a slaughterhouse. This was achieved by showing that all clinical isolates from the outbreak belonged to a single PFGE type and all other clinical isolates had a different PFGE type. In addition isolates from the pig slaughterhouse, its supply herds, isolates from pork and beef from the central marketplace that the

slaughterhouse supplied, as well as pork and some beef isolates from butcher shops supplied by the slaughterhouse during the outbreak were all shown to belong to the same PFGE type as the isolates of the affected humans. These results implicate the source of the outbreak as the pig herds supplying the slaughterhouse.

Another common application of REA and PFGE is population studies such as those investigating the genomic lineage of *Salmonella* serovars. Generally this is achieved by using REA in combination with other chromosomally based typing methods such as RFLP. In particular, Olsen & Skov (1994) and Olsen *et al.* (1994b, 1996) have employed this approach to study the evolution and relatedness of *S. enteritidis*, *S. dublin* and *S. gallinarum*.

1.2. e (ii) Genome probing

A method by which the number of restriction fragments analysed can be reduced is to transfer the DNA fragments from the gel after electrophoresis to a membrane and hybridize the membrane with a labelled probe (Olsen *et al.*, 1993). As with REA, a fingerprint for each strain is identified and related strains are grouped by similar fingerprints.

Various regions of the genome have been prepared as the labelled probe including randomly cloned fragments, specific regions of the genome such as 16S and/or 23S rRNA sequences (ribotyping) or insertion sequences such as IS*200* in *Salmonella* (IS*200* typing) (Olsen *et al.*, 1993). Restriction fragment length polymorphism (RFLP) analysis is another generic name given to the group of typing methods which involve genome probing (Olsen *et al.*, 1993, 1994b).

The label used on the probe can be of several types followed by the use of an appropriate detection system. Radioactive labels have been used as well as non-radioactive alternatives which include chemiluminescent, biotin and digoxigenin.

A consequence of methods such as these which analyse a smaller number of fragments is a loss in discriminatory power because only the heterogeneity of the restriction sites in a small region of the genome are considered, compared to REA in which the distribution of restriction sites throughout the entire genome is analysed (Prevost *et al.*, 1992; Schoonmaker *et al.*, 1992). An advantage of probing is that

PFGE is not required; the restriction enzyme digested genomic DNA can be electrophoresed on standard agarose gels prior to transfer to the membrane.

Ribotyping is a genome probing method in which the labelled probe is ribosomal RNA (rRNA) genes (Stull *et al.*, 1988). The probe is most commonly 16S and/or 23S rRNA from *E. coli*. However, Olsen *et al.* (1992a) demonstrated that ribosomal genes from bacteria other than *E. coli* are also suitable as probes. Comparison of the grouping obtained by ribotyping *S. berta* isolates with both an *E. coli*-derived probe and a fragment of the ribosomal genes of *Legionella pneumophila* showed that the same groupings resulted independent of the probe used.

Ribotyping has been used to investigate the relationships between strains for many *Salmonella* serovars including *S. reading*, *S. senftenberg*, *S. typhimurium*, *S. berta*, *S. dublin*, *S. enteritidis*, *S. infantis*, *S. typhi*, *S. gallinarum* and *S. pullorum* (Nastasi *et al.*, 1991; Olsen *et al.*, 1992a, 1994b; Christensen *et al.*, 1993; Esteban *et al.*, 1993; Olsen & Skov, 1994; Pelkonen *et al.*, 1994). However, ribotyping has never been used to investigate *S. virchow* strains.

IS*200* typing utilizes the near *Salmonella* specific IS*200* insertion sequence as the probe in this genome probing method. IS*200* is an insertion sequence or transposable element of 708 basepairs (bp) which is present in a large proportion of *Salmonella* serovars and *Shigella,* but not present in *E. coli* or other members of the *Enterobacteriaceae*. Lam and Roth (1983) first described IS*200* in *Salmonella*. Their study of the distribution of IS*200* included 15 *Salmonella* serovars and 11 other enteric bacteria. IS*200* was present in all but three of the *Salmonella,* which were *S. agona*, *S. arizonae* (monophasic) 7a, 7b,:1, 7, 8 and *S. arizonae* (diphasic) 26:23:31. Gibert *et al.* (1990) also confirmed that of 24 *Salmonella* serovars all but *S. agona* had at least one copy of IS*200*.

The study of Gibert *et al.* (1990) was the first to test strains of *S. virchow*; two strains were tested, one was characterized by one copy and the other strain by two copies. Inter and intra-serovar variation in the number of copies present were also observed (Gibert *et al.*, 1990). Generally, *S. typhi* was found to carry the highest number of copies at 10-25 but many serovars carried only two or three copies. *S. typhimurium* LT2 strain has been shown to have six copies and the approximate locations of the copies have been mapped using Tn*10* insertions (Sanderson *et al.*,

1993). In addition, some strains of *S. typhimurium* LT2 were shown to have a seventh copy (Sanderson *et al.* 1993).

Stanley *et al.* (1991) used the distribution of IS*200* within *S. enteritidis* as a molecular tool and demonstrated its ability to confirm clonal lineages for population studies. These studies also confirmed the epidemiological divisions based on phage typing.

IS*200* typing has since been applied to other serovars including *S. typhimurium*, *S. typhi*, *S. heidelberg* and *S. virchow* (Stanley *et al.*, 1992a; Baquar *et al.*, 1993; Torre *et al.*, 1993; Threlfall *et al.*, 1994). In comparison to the majority of other serovars the number of *S. virchow* strains with IS*200* is quite low. Torre *et al.* (1993) showed that only approximately 10% of the type strains of 57 different phage types of *S. virchow* had an IS*200* present. Of the 57 type strains tested, six carried IS*200* elements and amongst these there were three patterns. However, there was no single band carrying the IS*200* elements which was common to all of the patterns.

1.2. e (iii) PCR-based genome typing

Random amplification of polymorphic DNA (RAPD) is a PCR-based typing method which involves the use of several short random oligonucleotide sequences as primers in the one PCR reaction. These short primers generate a number of random PCR products due to the multiple annealing sites. A pattern is observed based on the number of bands and the size of the amplified products (Olsen *et al.*, 1993).

A second example of a PCR-based typing method is PCR ribotyping. This technique involves using specific primers which are complimentary to the highly conserved regions of the 16S and 23S rRNA genes and results in amplification of the 16S-23S spacer region (Kostman *et al.*, 1992, 1995; Lagatolla *et al.*, 1996). While the rRNA genes show a high level of homology, the intergenic spacer regions are highly variable and show variations in both sequence and length. The variation in the spacer region coupled with the fact that the rRNA loci are present in 2 to 11 copies on the chromosomes of most bacterial species result in distinctive patterns (Lagatolla *et al.*, 1996). The major advantage of this method is that it is applicable to many bacteria because the target is the conserved rRNA genes. Restriction digest of the PCR

products that result from this method can also be performed to attain a further level of discrimination.

Salmonellae isolates from ten different serovars, isolated from patients with diarrhoea, food and poultry in Italy were successfully typed by PCR ribotyping (Lagatolla *et al.*, 1996). Again, *S. virchow* was not included. Despite PCR ribotyping being successfully applied to *Salmonella* and numerous other bacterial genera there have been reports that it is not an effective method for all organisms. Cartwright (1995) reported that for both methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *E. faecium* this method was less discriminatory than PFGE. This was because relatively few bands were obtained by PCR ribotyping and all of the isolates of *E. faecium,* in particular, had an identical banding pattern. In this situation PCR ribotyping was of no typing value.

1.2. h) Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MEE) involves analysis of allelic variation in soluble metabolic enzymes. This technique has been applied to the study of populations and is able to determine the degree of genetic diversity in a population.

MEE is a labour intensive and a time consuming typing method but it can have significant discriminatory power. Briefly the method involves producing cell free extracts of cultures using sonication, to obtain the soluble metabolic enzymes. The cell lysates are electrophoresed on starch or polyacrylamide gels using a range of buffering systems (Seltmann *et al.*, 1994; Cox *et al.*, 1996b). The individual enzymes are localized on the gels after electrophoresis using specific staining methods such as those described by Selander *et al.* (1986). Different alleles of each enzyme are identified by their mobility in the gels and each different allele, or electromorph, is given a number. A profile for each isolate is determined by combining the electromorphs for each of the different enzymes tested and each distinct profile is an electrotype (ET). The genetic distance between isolates can then be determined by considering statistically the number of loci, or enzymes, at which dissimilar alleles occur (Cox *et al.*, 1996b).

MEE has been applied to *Listeria monocytogenes* (Nørrung & Gerner-Smidt, 1993; Graves *et al.*, 1994; Boerlin *et al.*, 1995), *E. coli* (Boyd *et al.*, 1994; Ngeleka *et al.*, 1996) and many *Salmonella* serovars including *S. typhi*, *S. enteritidis*, *S. cholerasuis*, *S. derby*, *S. dublin*, *S. heidelberg*, *S. infantis*, *S. newport* and *S. typhimurium* (Beltran *et al.*, 1988; Reeves *et al.*, 1989; Seltmann *et al.*, 1994; Cox *et al.*, 1996b). It has not yet been applied to *S. virchow*.

1.3. Bacterial Virulence Factors of Salmonella

A virulence factor refers to a specialized feature of a bacterium which it uses to enter a host or colonize a host (Dorman, 1994). Virulence factors also have a role in evading the host's defence mechanisms or in excluding other competing organisms. Generally each factor is involved at a particular stage of the interaction between the organism and the host and not all bacteria employ all of the factors, depending on the type of infection they cause (Dorman, 1994). Some of the virulence factors for which a mechanism has been established include adhesins, capsules and fimbriae. The two virulence factors which are dealt with in this thesis, fimbriae and lipopolysaccharide, are discussed in detail as an introduction to the experimental sections and several other virulence factors are briefly discussed.

1.3. a) Fimbriae

Fimbriae are virulence - associated proteinaceous surface structures which have been demonstrated to mediate adhesion of bacterial cells to host tissues and in some cases also facilitate colonization (Tarkkanen *et al.*, 1990; Collinson *et al.*, 1993; Kukkonen *et al.*, 1993).

Within the *Enterobacteriaceae*, and in particular *E. coli*, fimbriae have been very well characterized. The type 1 fimbriae which are responsible for attachment have been extensively studied. Some of this knowledge has lead to the development of diagnostic tests and subunit vaccines.

The association between the expression of fimbriae by *Salmonella* (as observed by electron microscopy) and the ability to agglutinate certain red blood cells was first made in 1958, but only more recently have many of the fimbriae been identified and characterized. Primary differentiation of fimbriae relies on the ability of fimbriae to mediate erythrocyte agglutination and whether the agglutination is D - mannose sensitive or resistant (Thorns, 1995).

There have been many fimbrial types identified in *Salmonella* serovars. The most studied of these is the Type 1 fimbria or SEF21. These are rigid structures, approximately 7nm in diameter. Type 1 fimbriae can be 100nm in length and there are as many as 300 per cell, although there may be only 10% of the cells expressing fimbriae at any one time. These fimbriae are made up of protein subunits of 20 - 22 kDa, linked around a hollow core which gives them a channelled appearance by electron microscopy (Müller *et al.*, 1991). These fimbriae mediate mannose - sensitive haemagglutination and bind to oligomannose side chains of laminin, alone or as part of a basement membrane (Duguid *et al.*, 1966; Kukkonen *et al.*, 1993). Within the salmonellae the structure and sequences of type 1 fimbriae are largely conserved (Müller *et al.*, 1991). The type 1 fimbriae of *S. enteritidis* and *S. typhimurium* also show some sequence homology to the type 1 fimbriae of other members of the *Enterobacteriaceae* (Purcell *et al.*, 1987).

Type 2 fimbriae are morphologically similar to type 1 and several workers have considered them to be a non-agglutinating variant of type 1 fimbriae (Clegg & Gerlach, 1987). This has some support because recombinant plasmids have been used to show that the structural and heamagglutination components of type 1 fimbriae in *E. coli* are encoded by separate genes (Minion *et al.*, 1986). This type 2 fimbriae was first observed in *S. gallinarum* and *S. pullorum* (Duguid & Gillies, 1958) but has also been described on isolates of *S. paratyphi* B and *S. dublin* (Duguid *et al.*, 1966)

Type 3 fimbriae are thinner and more flexible than Types 1 and 2, with a diameter of 3-5nm. These fimbriae agglutinate tannic acid - treated erythrocytes in the presence of α-D-mannose and bind to human type V collagen (Duguid *et al.*, 1966; Tarkkanen *et al.*, 1990).

Type 4 fimbriae, only recently described from an isolate of *S. typhimurium* mediate mannose - resistant agglutination of fresh erythrocytes (Grund & Weber, 1988; Grund & Seiler, 1993). These fimbriae are defined as thin and flexible with a diameter of 4nm. There are several differences between this and other fimbrial types. Type 4 are the only fimbriae so far identified which do not contain signal sequences which are cleaved by signal peptidase I. Instead, type 4 fimbriae have a different export system and contain signal sequences which are cleaved by type IV pre-pilin signal peptidase (Bäumler & Heffron, 1995). Also type 4 fimbriae are polar in their

arrangement in contrast to the other fimbriae which are peritrichous. Type 4 fimbriae are also a different length at 10 to 20 μm compared with 0.5 to 2 μm for other fimbriae (Bäumler & Heffron, 1995).

SEF14 and SEF17 are two fimbrial types which do not fit into the existing classification system because they do not agglutinate erythrocytes. SEF14 are thin fimbriae with a diameter of <3nm which were first seen on *S. enteritidis* (Thorns *et al.*, 1990). These fimbriae are composed of protein subunits of 14.3kDa and are specific to *Salmonella* serovars in Group D. All *S. enteritidis* isolates and some *S. dublin* isolates investigated by Thorns *et al.* (1992) showed these fimbriae. The structural gene was cloned and sequenced and shown to be present in *S. blegdam*, *S. dublin*, *S. enteritidis*, *S. gallinarum*, *S. moscow*, *S. pullorum*, *S. rostock*, *S. seremban* and *S. typhi*, all of which belong to group D. However SEF14 antigen was shown to only be expressed in *S. dublin*, *S. enteritidis*, *S. blegdam* and *S. moscow* (Turcotte & Woodward, 1993).

The operon which contains the genes encoding the SEF14 fimbria of *S. enteritidis* is perhaps the most well characterized. The genes *sefABCD* are an operon which encodes synthesis and assembly of SEF14 fimbriae. *sefA* is the gene for the structural SEF14 fimbrial subunit. *sefB* encodes transport proteins similar to *E. coli* chaperone proteins and *sefC* encodes usher proteins in the outer membrane. These usher proteins are thought to play a role in assembly of subunits into functional fimbriae (Thorns, 1995).

sefD is a fourth open reading frame which overlaps *sefC*. A consequence of the *sefC* and *sefD* genes overlapping is that *sefD* was characterized as a member of the SEF14 gene cluster. Therefore, this cluster is the first fimbrial gene cluster in *Enterobacteriaceae* to encode two fimbrin - like proteins which form two distinct cell surface structures. Although this is unknown in the *Enterobacteriaceae* it has been seen in other bacteria (Clouthier *et al.*, 1994).

The translated protein sequence of *sefD* is unique but similar to the genes of other fimbriae which encode structural proteins. *sefD* was defined as the major gene of a new fimbriae - like structure, SEF18 (Clouthier *et al.*, 1994). SEF18 are not yet confirmed fimbriae as electron microscopy has not identified distinct fibres and the protein has not been purified (Clouthier *et al.*, 1994). The function of these fimbriae is also as yet unknown but they are biochemically and serologically distinct from SEF14,

17 and 21. Immuno-gold electron microscopy with polyclonal immune sera raised to affinity-purified recombinant *sefD* demonstrated filamentous fimbriae - like structures often concentrated at the junctions between cells and so it was hypothesized that they may be involved in cell-cell adherence. The *sefD* gene was PCR - amplified and used as a genomic probe to determine the distribution of this gene within *Salmonella* and other *Enterobacteriaceae*. It was found that the DNA sequence of *sefD* is widely distributed, because the probe bound to all of the *Salmonella* DNA tested (*S. virchow* was not included) and many other members of the *Enterobacteriaceae*. The sequence was also found to be more highly conserved than the sequences encoding the type 1 fimbriae (Clouthier *et al.*, 1994).

The first report of plasmid-encoded fimbriae was made by Friedrich *et al.* (1993). The *pef* (plasmid - encoded fimbriae) locus was located on the 90kb plasmid of *S. typhimurium* and this plasmid conferred increased virulence in mice. A 7kb region was shown to contain seven open reading frames of which five gave protein products related in sequence to regulatory, structural and assembly proteins of adherence fimbriae such as K88 of *E. coli* and SEF14 (Friedrich *et al.*, 1993).

The most recently identified fimbriae of *Salmonella* are SEF17. These fimbriae were first described on *S. enteritidis* in 1991 by Collinson *et al.* (1991), who were investigating fimbriae of *S. enteritidis* 27655-3b. Like SEF14, the SEF17 fimbriae do not fit into the fimbria classification scheme because they do not agglutinate erythrocytes (Thorns, 1995). During immunogold labelling studies of the previously characterized SEF21 and SEF14, a third and morphologically distinct fimbrial type was observed on *S. enteritidis* 27655-3b when grown at 30°C (Müller *et al.*, 1991). These thin (3-4nm in diameter), aggregative fimbriae were purified using an unconventional method requiring pretreatment with 90% formic acid, to enable the protein to migrate into polyacrylamide stacking gels and then through the resolving gel. Western blot analysis with immune serum showed the major immunoreactive protein was 17kDa (Collinson *et al.*, 1991).

By 1992 some *E. coli* strains had been shown to produce fimbriae which were both morphologically and antigenically related to the SEF17 of *S. enteritidis* 27655-3b (Collinson *et al.*, 1992). These fimbriae were made up of an 18kDa fimbrin, encoded by *csgA,* which also required depolymerization with formic acid in order to undergo

electrophoresis. Western blotting demonstrated that the *E. coli* fimbrin cross - reacted with the rabbit immune serum raised against purified SEF17. Therefore, the group of thin aggregative fimbriae, identified in two genera of enteropathogens, was given the general name GVVPQ fimbriae because of the N-terminal amino acid sequence homology shared (Collinson *et al.*, 1992).

Collinson *et al.* (1993). showed that SEF17 were responsible for binding of fibronectin by *S. enteritidis* 27655-3b. This strain was also able to bind collagen types I and IV, a capacity which had not been shown previously in *S. enteritidis*

With the structure and function of SEF17 becoming clear, Collinson *et al.* (1993) reported screening 113 isolates, representing 95 serovars of *Salmonella,* and several other members of the *Enterobacteriaceae* and eubacteria for GVVPQ fimbriae by Congo red binding. Positive isolates were then subjected to Western blotting using antiserum to SEF17. However, because of the highly variable production of these fimbriae, a genotypic approach was also used. A section of the gene, *agfA,* which encodes the SEF17 fimbrin, was amplified by PCR and used as a radioactively - labelled probe. The results showed that *agfA* is widely distributed in *Salmonella* spp.. No *S. virchow* isolates were included in the study. It should be noted that although there is significant sequence homology between the fimbrin genes of *S. enteritidis* (*agfA*) and *E. coli* (*csgA*), there is sufficient dissimilarity that the *agfA* probe only hybridized to *Salmonella* DNA (Collinson *et al.*, 1996a).

Presently it is unknown how many genes are involved in the biosynthesis of SEF17 but Collinson *et al.* (1996a) have identified more open reading frames upstream and downstream of *agfA*. The operon, *agfBAC* consists of *agfB* which is upstream of *agfA*. The predicted amino acid sequence of *agfB* matches that of *agfA* in size and primary sequence and it would appear that *agfB* encodes a fimbrin - like protein with a signal sequence characteristic of an exported protein (Collinson *et al.*, 1996a). *agfC* is the third open reading frame and it is downstream of *agfA* but under the control of the same transcriptional start site as *agfA* and *agfB*. No transcripts of *agfC* have so far been detected (Collinson *et al.*, 1996a).

Recently the gene clusters of *agfA* (SEF17), *fim* (SEF21), and *sefABCD* (SEF14 and SEF18) were mapped onto the *Xba*I-*Bln*I restriction maps of *S. typhimurium* LT2 and *S. enteritidis* strains 27655-3b and SSU7998. This demonstrated that all of these

genes are chromosomally encoded and are widely separated on the chromosome (Collinson *et al.*, 1996b).

1.3. b) Lipopolysaccharides

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. It plays an important role in the proper organization and functioning of the bacterial cell outer membrane (Reeves, 1994). LPS is also a receptor for bacteriophages and antibodies which allows host defences to identify and eliminate bacterial cells. However, LPS also plays a role in shielding pathogens from cellular host defences and thus is a key virulence factor. LPS is able to prevent activation of the complement cascade and uptake of bacterial cells by phagocytic cells and when purified has toxic effects and is pyrogenic (Morrison & Ryan, 1992).

LPS is composed of distinct regions namely, lipid A, core oligosaccharide (which has inner and outer regions) and O-polysaccharide or O-antigen. LPS consisting of all three components is called smooth or wild type LPS. However some Gram-negative bacteria do not synthesize the O-specific polysaccharide chain. This results in an LPS molecule which is termed R type LPS (for rough mutants) (Morrison & Ryan, 1992). Generally rough mutants are non-pathogenic and often serum sensitive but some pathogens including *Bordetella pertussis*, *Campylobacter jejuni* and *Haemophilus influenzae* remain virulent despite having R type LPS (Reeves, 1994).

The three major components of LPS are synthesized individually and then assembled. The sequence of events in the biosynthesis of LPS is:

1. Lipid A is synthesized. Lipid A is highly conserved and so it is speculated that the biosynthetic pathways involved are similar for many species (Reeves, 1994);
2. Core oligosaccharide is synthesized by sequential addition of sugars onto the lipid A by enzymes encoded by the *rfa* genes (Palva & Mäkelä, 1980). All of the steps in biosynthesis of the lipid A/core are carried out with the molecule anchored to the cytoplasmic membrane;

3. Assembly of the repeating oligosaccharide units of the O-antigen occurs simultaneous to the other events, but independently. This begins with transfer of galactosyl-1-phosphate to the lipid carrier undecaprenyl phosphate, by a transferase. Subsequently other sugar residues are added sequentially by specific sugar transferases. The *rfb* gene cluster encodes the enzymes involved in this pathway. The sugars included and the sequences of assembly are dependent on the serovar and this sequence information is known for many serovars (Wang & Reeves, 1994);
4. The assembled O-units are then polymerized to form the O-polysaccharide side chain or O-antigen. This is catalysed by the enzyme O polymerase which is encoded by *rfc*. There is some heterogeneity present at this stage as the number of single O-units which are polymerized to create the O-polysaccharide can vary. The side chain is of a preferred length and some control is maintained over this length by the gene product of *cld* (chain length determinant) (Reeves, 1994);
5. The O-polysaccharide side chain is then transferred from the lipid carrier to the lipid A/core oligosaccharide. If the complete core oligosaccharide is not present (as in *rfa* mutants) the transfer from lipid carrier to lipid A/core does not occur;
6. Lastly, some post assembly modifications are made including the addition of glucosyl or other branch structures.

The LPS structure may exhibit considerable variation and some heterogeneity occurs in all of the three major components. This heterogeneity in the biochemistry of the LPS can be detected by differences in profiles on polyacrylamide gels (Hitchcock & Brown, 1983; Reeves, 1994).

Lipid A has the least heterogeneity but publications by Rosner *et al.* (1979a, b) described variations in the fatty acid composition of lipid A. There is also some variation in the core oligosaccharide structure. These differences can be detected as changes in the relative mobility of the leading band (Hitchcock & Brown, 1983).

The third component, the O-specific polysaccharide or O-antigen shows the most heterogeneity (Helander *et al.*, 1992; Reeves, 1994). Between serogroups, serovars and strains there are differences in the sugars present and the sequence of the

sugars which make up the O-units and also the number of repeating units in any given O-side chain (Palva & Mäkelä, 1980; Reeves, 1994). There is also further heterogeneity observed as distinct banding patterns when the LPS is electrophoresed on a polyacrylamide gel. Sometimes the bands of the repeating sugar units in the O-side chain appear as singlet bands and sometimes as doublets (Palva & Mäkelä, 1980; Chart & Rowe, 1995). Intra-strain heterogeneity in *S. virchow* was recently described in which different colonies of the same strain exhibited different profiles of the repeating units (Chart & Rowe, 1995). Fifty percent of the colonies showed the doublet appearance and the other 50% produced single bands and it was proposed that this stems from two different pathways of O-antigen synthesis.

Some of the factors which have been shown to influence LPS structures are growth conditions such as temperature, the medium and the presence of plasmids. McConnell & Wright (1979) reported that a *S. anatum* strain which synthesized smooth LPS when grown at 37°C underwent a partial smooth-rough transition if grown at 20-25°C. The growth at lower temperatures resulted in a greater proportion of LPS molecules with core oligosaccharide unsubstituted with O-antigen (rough type). Palva & Mäkelä (1980) also reported different profiles depending on whether cells were grown on minimal or complete media. When complete medium was used the upper band of the doublets was most intense when compared with those from cells grown on minimal medium.

Phage type has also been shown to be closely associated with LPS expression and virulence of *Salmonella* serovars (Chart *et al.*, 1991b, 1993). Isolates of *S. enteritidis* phage type 4 convert to phage type 7 spontaneously with complete loss of expression of long chain LPS. Chart *et al.* (1991b) demonstrated that *S. enteritidis* strains belonging to phage types 7, 23 and 30, which did not express long chain LPS, were avirulent for BALB/c mice. This highlighted the link between phage type, LPS and virulence.

Chart *et al.* (1991a) also showed that phage type alone does not dictate the virulence of strains. Despite all strains of *S. enteritidis* phage types 8, 13a and 24 having long-chain LPS, one strain of phage type 13a and two strains of phage type 24 were avirulent for BALB/c mice. Plasmid analysis revealed that all strains which were virulent for mice contained a 38 MDa plasmid. The avirulent strain of phage type 13a

was plasmid-free and the avirulent strains of phage type 24 carried a 34 MDa plasmid which was shown to be significantly different to the 38 MDa plasmid by restriction analysis. In this case the presence of a plasmid altered the virulence of strains but differences in the LPS profiles of strains with and without plasmid were not apparent. This is not the case for a strain of *S. dublin* reported by Kawahara *et al.* (1989). In this strain, an ca. 50MDa virulence plasmid was shown to encode factors which alter LPS expression. A virulence plasmid-cured derivative exhibited semi rough LPS while a strain with the re-introduced virulence plasmid exhibited smooth LPS. The same effect was not seen with other *S. dublin* strains or strains of other serovars and so it was concluded that the genes located on this plasmid were not structural genes of enzymes involved in LPS biosynthesis but encoded a regulatory factor (Kawahara *et al.*, 1989). However, for a strain of *S. borreze,* a 6.7kb plasmid encodes the enzymes responsible for the synthesis of the O:54 antigen (Stocker & Mäkelä, 1971). Curing of this plasmid resulted in loss of long-chain LPS structure and the O:54 serotype. Usually, the O:54 polysaccharide is also chromosomally encoded in these strains and the polysaccharide is co-expressed.

To date only Chart & Rowe (1995) have reported investigating the LPS structure of *S. virchow.* Their findings showed that *S. virchow* expresses two distinct LPS structures within a strain. This was the first description of such heterogeneity in LPS expression. The distinct structures referred to were the single runged or double runged appearance of the LPS O-side chain after polyacrylamide gel electrophoresis and silver staining. The different profiles were not linked to either the phage type or the source of isolation and the strains tested comprised 28 different phage types of *S. virchow* of either human or animal origin. This difference in profiles reflected differences in the O-side chains and the conclusion was that *S. virchow* strains have more than one pathway for assembly of LPS chains.

1.3. c) Virulence plasmids

Jones *et al.* (1982) were the first to describe the association between the high molecular weight 'cryptic' plasmid of *S. typhimurium* and virulence. Since then high molecular weight plasmids have been shown to be responsible for the virulence of many non-typhoid serovars associated with systemic infection including *S. cholerasuis*, *S. dublin*, *S. enteritidis* and *S. typhimurium* (Gulig & Curtiss III, 1987; Kawahara *et al.*, 1990; Lax *et al.*, 1990; Buisán *et al.*, 1994). Although the plasmids are different between serovars, ranging in size from 50-100kb, they share a homologous region of 8kb, which contains the *spv* locus (Guiney *et al.*, 1995). The *spv* locus includes the *spvR* regulatory gene and four structural genes, *spvABCD*.

Most of the investigations of virulence and the role of the virulence plasmids have focussed on experimental infection of mice and the observation of different levels of virulence by wild type strains, cured strains or cured strains in which the virulence plasmid had been reintroduced (Pardon *et al.*, 1986; Gulig & Curtiss III, 1987; Beninger *et al.*, 1988). Heffernan *et al.* (1987) used experimental infection in mice to show that the plasmid is not needed for invasion of the bacteria into intestinal epithelial cells and Peyer's patches. Neither was it required for the spread to mesenteric nodes. Instead the plasmid-containing strains were able to outgrow plasmid-free strains in the reticuloendothelial system. The consequence of this outgrowth is an infection involving large numbers of organisms in the liver and spleen.

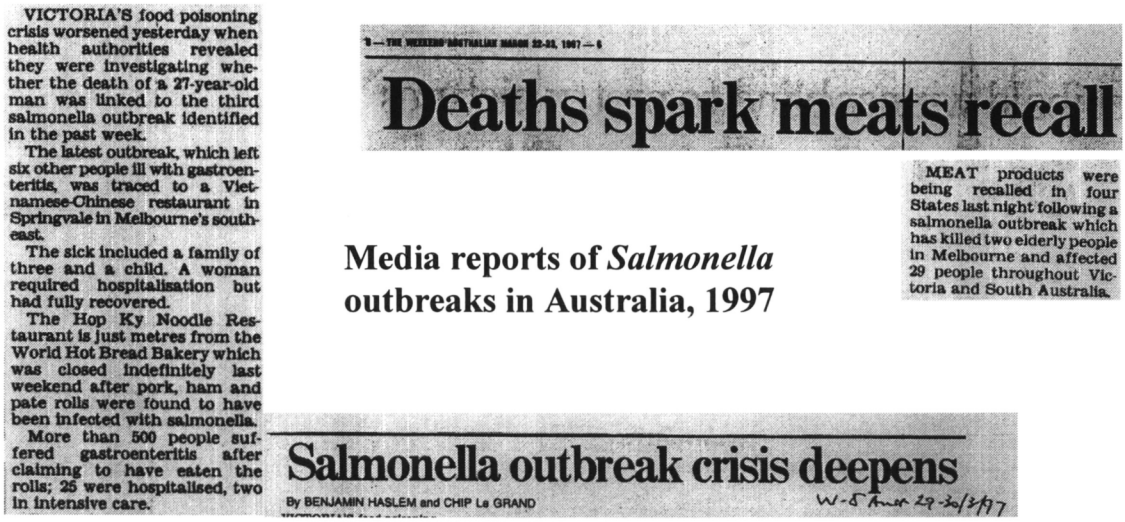
More recently, molecular approaches such as cloning and the use of protein expression systems have allowed protein products of each of the *spv* genes to be identified (Gulig *et al.*, 1993). Also the expression and regulation of the virulence genes is being elucidated. Studies by Guiney *et al.* (1995) indicated that induction of the *spv* genes occurs in intracellular environments and that the alternative σ factor, RpoS, and SpvR (protein product of *spvR*) together regulate the expression of the *spv* operon.

Despite *S. virchow* belonging to the group of non-typhoid *Salmonella* serovars which can cause systemic infections, no *S. virchow* strains have been reported to have a serotype-specific virulence plasmid.

1.3. d) Other virulence factors of *Salmonella*

There are many virulence factors that are employed by various pathogens including *Salmonella* and undoubtedly many that are yet to be identified and characterized. Some of the known factors are adhesins (other than fimbriae which have been discussed) such as mannose-resistant haemagglutinin and certain invasion proteins (Finlay & Falkow, 1989). Toxins and heat shock proteins are also virulence factors that have been associated with *Salmonella* serovars (Wallis *et al.*, 1986; Buchmeier & Hefron, 1990; Johnson *et al.*, 1991; Malik *et al.*, 1996). The heat shock proteins are perhaps inappropriately named as it is not only heat that induces expression of heat shock proteins but many other stresses generally associated with changes in the local environment of bacterial cells. Some of the stimuli or stresses that induce heat shock proteins include acidic pH, reactive oxygen intermediates such as hydrogen peroxide and hydroxyl radicals, iron depletion and heavy metals (Morgan *et al.*, 1986; Hickey & Hirshfield, 1990; Kaufmann & Flesch, 1992; Bearson *et al.*, 1996).

1.4. Objectives



VICTORIA'S food poisoning crisis worsened yesterday when health authorities revealed they were investigating whether the death of a 27-year-old man was linked to the third salmonella outbreak identified in the past week.

The latest outbreak, which left six other people ill with gastroenteritis, was traced to a Vietnamese-Chinese restaurant in Springvale in Melbourne's southeast.

The sick included a family of three and a child. A woman required hospitalisation but had fully recovered.

The Hop Ky Noodle Restaurant is just metres from the World Hot Bread Bakery which was closed indefinitely last weekend after pork, ham and pate rolls were found to have been infected with salmonella.

More than 500 people suffered gastroenteritis after claiming to have eaten the rolls; 25 were hospitalised, two in intensive care.

Deaths spark meats recall

MEAT products were being recalled in four States last night following a salmonella outbreak which has killed two elderly people in Melbourne and affected 29 people throughout Victoria and South Australia.

Salmonella outbreak crisis deepens

By BENJAMIN HASLEM and CHIP Le GRAND

Media reports of *Salmonella* outbreaks in Australia, 1997

In the Australian state of Victoria, outbreaks of several different *Salmonella* serovars associated with foods, occurred early in 1997. The application of typing methods is an important aspect of investigations of such outbreaks. Typing of strains isolated during an outbreak is used to confirm epidemiological data and identify the source of the outbreak, the vehicle of transmission and to distinguish between outbreak cases and sporadic cases.

In light of this ongoing need for discriminatory typing methods the first aim of this study is to apply several typing methods including antimicrobial susceptibility testing, plasmid profiling, phage typing and ribotyping, to a group of *S. virchow* isolates, to establish the discriminatory power of each of these methods and their potential to type *S. virchow*.

Research in the United Kingdom and Netherlands has shown that poultry is a major source of human *Salmonella* infection. In Australia, the link between poultry and humans has not been demonstrated for *Salmonella*. Therefore, an objective is to determine if poultry is a source of *S. virchow* infection in humans in Australia. The combination of the results from several typing methods should allow closely related strains to be identified. Strains which are indistinguishable will represent clonal lines of *S. virchow*. Clonal lines which contain both human and poultry isolates would

provide strong evidence that poultry is a vehicle of transmission of *S. virchow* to humans in Australia.

In addition to the epidemiological studies, potential virulence factors of *S. virchow* are also to be investigated. *S. virchow* has been associated with extra-intestinal infections in humans and the SEF17 fimbriae and lipopolysaccharide have been chosen for detailed investigation. Both of these structures have been shown to be important in the virulence of *S. enteritidis*.

A third major area of this study is concerned with methodology. A system to study virulence which does not rely on mouse models and rabbit loop tests would be desirable for both technical and ethical reasons. A generic invasion assay is widely referred to in the literature and the aim of this section is to evaluate the performance of *S. virchow* in this assay. The application of such an assay would be to predict whether a *S. virchow* isolate is capable of invasive infection. In addition, if the assay is found to be adequately sensitive it could be used, instead of mouse models, to observe the effects of genetic manipulation of virulence factors.

CHAPTER 2

EPIDEMIOLOGICAL STUDY OF

S. VIRCHOW

Chapter 2 : Epidemiological Study of *S. Virchow*

2.1 Introduction

The epidemiological investigation of *S. virchow* was designed with three objectives. The first was to apply several typing methods to *S. virchow* isolates and determine the level of discrimination for each.. Although records of the incidence of *Salmonella* serotypes in Australia are available from the National *Salmonella* Surveillance Scheme there has not been a study in which several genotypic and phenotypic typing methods have been applied to *S. virchow.*

The second objective of this study was to determine if any clonal relationships were present between *S. virchow* isolates from Australia. Clonal relatedness between different isolates can be shown by demonstrating the presence of common characteristics by the use of typing methods. Clones have been defined as strains isolated independently but which exhibit so many identical phenotypic and genotypic traits that the most likely explanation is that they come from a common ancestor (Orskov & Orskov, 1983). This definition allows some flexibility because no actual number of identical traits required is stated. The combination of results from all five typing methods would identify isolates that were very closely related, either by source, date of isolation, or by evolutionary origin and thereby reveal clonal lines of *S. virchow.*

The third objective was to determine if the clonal relationships between isolates of *S. virchow* confirmed the hypothesis that poultry is a major vehicle of transmission of infection to humans. Poultry has been identified as the vehicle for transmission of *S. typhimurium, S. enteritidis* and *S. virchow* to humans in the United Kingdom and the Netherlands (Humphrey *et al.*, 1988; Reilly *et al.*, 1988; Giessen *et al.*, 1991). The occurrence of indistinguishable strains of *S. virchow* in both the poultry and human populations of Australia would indicate a causal link between the two groups.

2.2 Materials and Methods

2.2. a) Bacterial strains and media

The *Salmonella* isolates examined in this study were obtained from the following institutions :-

- Institute of Medical and Veterinary Science (IMVS) - Adelaide
- Medical Diagnostic Unit (MDU) - Department of Microbiology, University of Melbourne
- State Health Queensland (SHQ)
- Queensland Department of Primary Industry (DPI) - Oonoonba, Townsville
- *Salmonella enteritidis* Egg Industry and Research Development Council farm survey project (farm survey)
- Princess Alexandra Hospital (PAH) - Queensland
- Australian Collection of Microorganisms (ACM) - Department of Microbiology, University of Queensland
- Royal Melbourne Institute of Technology (RMIT)

Table 2.1 provides details of the *S. virchow* isolates and their original source and Table 2.2 contains the details of all other *Salmonella* and non - *Salmonella* strains.

Table 2.1 - Origins of *S. virchow* isolates

Isolate Number	Source	Other details (Provided by)
1	meat/bone meal	Qld (IMVS)
2	frog's legs	imported (IMVS)
3	possum	- (IMVS)
4	human	acquired overseas (IMVS)
5	macadamia nuts	- (IMVS)
7	human urine/faeces?	- (PAH)
8	chicken meat	Qld Oct 1991 (IMVS)
9	chicken meat	Qld Sept 1991 (IMVS)
11	chicken meat	Qld Sept 1991 (IMVS)
12	chicken meat	Qld Sept 1991 (IMVS)
13	chicken meat	Qld Sept 1991 (IMVS)
14	chicken meat	Qld Sept 1991 (IMVS)
15	chicken meat	Qld Sept 1991 (IMVS)
16	chicken meat	Qld Sept 1991 (IMVS)
17	chicken meat	Qld Sept 1991 (IMVS)
18	chicken meat	Qld Sept 1991 (IMVS)
19	chicken meat	Qld Sept 1991 (IMVS)
20	chicken meat	Qld Sept 1991 (IMVS)
21	chicken meat	Qld Sept 1991 (IMVS)
22	chicken meat	Qld Sept 1991 (IMVS)
23	chicken meat	Qld Oct 1991 (IMVS)
24	chicken meat	Qld Oct 1991 (IMVS)
25	chicken meat	Qld Oct 1991 (IMVS)
26	chicken meat	Qld Oct 1991 (IMVS)
27	chicken meat	Qld Oct 1991 (IMVS)
28	chicken meat	Qld Oct 1991 (IMVS)
29	chicken meat	Qld Oct 1991 (IMVS)
30	chicken meat	Qld Nov 1991 (IMVS)
31	human urine/faeces?	- (PAH)
33	chicken faeces	18/5/92 (farm survey)
34	feed component -full fat soya bean	13/4/92 (farm survey)
35	human faeces	Bundaberg Qld May 1992 (IMVS)
36	human faeces	Grantham Qld May 1992 (IMVS)
37	human faeces	Brisbane Qld June 1992 (IMVS)
38	human faeces	Wacol Qld Feb 1992 (IMVS)

Isolate Number	Source	Other details (Provided by)
39	human faeces	Gladstone Qld May 1992 (IMVS)
40	human faeces	Rockhampton Qld June 1992 (IMVS)
41	chicken meat	Qld May 1992 (IMVS)
42	chicken meat	Qld May 1992 (IMVS)
43	sewage effluent	Penrith NSW April 1992 (IMVS)
44	sewage effluent	Penrith NSW April 1992 (IMVS)
45	sewage effluent	Penrith NSW April 1992 (IMVS)
46	sewage effluent	Sydney NSW June 1992 (IMVS)
47	chicken faeces	18/5/92 (farm survey)
48	chicken faeces	21/4/92 (farm survey)
50	human faeces	Rockhampton Qld Oct 1992 (IMVS)
51	human faeces	Port Douglas Qld Nov 1992 (IMVS)
52	human faeces	Cairns Qld Aug 1992 (IMVS)
53	human faeces	Cairns Qld Sept 1992 (IMVS)
54	human faeces	Rockhampton Qld Oct 1992 (IMVS)
55	human faeces	Adelaide SA Nov 1992 (IMVS)
56	human faeces	Adelaide SA Oct 1992 (IMVS)
57	urine	Rockhampton Qld Nov 1992 (IMVS)
58	seawater	Bali Indonesia (sent from WA) 1992 (IMVS)
59	human faeces	Newcastle NSW Oct 1992 (IMVS)
60	bovine faeces	16/3/82 (DPI)
61	Agile wallaby-ileocaecal lymph node	27/1/83 (DPI)
62	Agile wallaby-ileocaecal lymph node	20/7/83 (DPI)
63	Black strip wallaby liver	3/4/85 (DPI)
64	Kangaroo faeces - domestic roo	Mungdinburra 29/5/85 (DPI)
65	Equine joint fluid	Hervey's Range 16/9/85 (DPI)
66	Grey Kangaroo faeces - domestic roo	Bohle 23/7/86 (DPI)
67	Bovine duodenum	Mareeba 23/7/86 (DPI)
68	Porcine Pus	Alice River 23/7/86 (DPI)
69	Cane toad faeces	Qld/NT 30/6/88 (DPI)
71	human urine/faeces?	1993 (PAH)

Isolate Number	Source	Other details (Provided by)
72	human faeces	Mackay Qld Jan 1993 (IMVS)
73	human faeces	Bald Hills Qld Jan 1993 (IMVS)
74	human faeces	Petrie Qld Jan 1993 (IMVS)
75	chicken liver/heart	Qld Jan 1993 (IMVS)
76	chicken meat	Qld Dec 1992 (IMVS)
77	human faeces	Farmborough Heights NSW Jan 1993 (IMVS)
78	chicken meat	Qld Feb 1993 (IMVS)
79	human faeces	Cairns Qld Jan 1993 (IMVS)
80	chicken meat	Qld Jan 1993 (IMVS)
81	human faeces	Beaudesert Qld Jan 1993 (IMVS)
82	female human faeces	Mackay Qld (MDU)
83	20 YO male human faeces	Sunbury Vic 25/9/92 no travel details (MDU)
84	1 YO female human faeces	Kingston Tas - travel to Fiji (MDU)
85	25 YO human faeces	Caulfield Vic (MDU)
86	42 YO male human faeces	Canberra ACT (MDU)
87	24 YO male human faeces	Bayswater Vic travel to Africa (MDU)
88	7 YO female human faeces	Chinchilla Qld (MDU)
89	80 YO male human faeces	Dalby Qld (MDU)
90	23 YO male human faeces	Noble Park Vic (MDU)
91	1 YO male human faeces	Maryborough Qld (MDU)
92	blood - male	Fiji (MDU)
93	67 YO male human faeces	Mackay Qld (MDU)
94	28 YO male human faeces	Mackay Qld (MDU)
95	2 YO female human faeces	Beenleigh Qld (MDU)
96	18 month male human faeces	Sandringham Vic (MDU)
97	3 YO male hipplate & screws	Noosa Waters Qld (MDU)
98	sheep faeces	Gnarwarre Vic (MDU)
99	human faeces	Maryborough Qld (MDU)
100	20 YO male human faeces	Sunbury Vic 1/3/93 (MDU)
101	18 month male human faeces	Mackay Qld (MDU)
102	55 YO female human faeces	Morayfield 9/3/95 (SHQ)
103	6 month male human faeces	Glenmore Grove 26/3/95) (SHQ)
104	10 month male human faeces	Lake Placid 20/3/95 (SHQ)
105	9 month male human faeces	Jimboomba 3/4/95 (SHQ)

Isolate Number	Source	Other details (Provided by)
106	70 YO female human urine	Eagleby 3/4/95 (SHQ)
107	14 YO male human urine	Lockrose 5/4/95 (SHQ)
108	70 YO female human urine	Eagleby 22/4/95 (SHQ)
109	44 YO male chest wall	Chatswood Hills 29/4/95 (SHQ)
110	7 month male human faeces	Glenmore Grove 31/4/95 (SHQ)
111	9 YO female human faeces	Jimboomba 3/5/95 (SHQ)
112	7 YO male human faeces	Surfers Paradise 21/4/95 (SHQ)
113	25 YO male human faeces	Mareeba 4/5/95 (SHQ)
114	9 month male human faeces	Tully Heads 3/5/95 (SHQ)
115	5 month female human faeces	Atherton 13/5/95 (SHQ)
116	1 YO male human faeces	Chambers Flat 22/5/95 (SHQ)
117	1 YO male human faeces	Chambers Flat 22/5/95 (SHQ)
118	unpasteurized frozen whole egg pulp	- (farm survey)
119	unpasteurized frozen whole egg pulp	- (farm survey)
120	chicken faeces	25/7/95 (farm survey)
121	human faeces	Feb 1996 case study (SHQ)
122	human faeces	Feb 1996 case study (SHQ)
123	human faeces	Feb 1996 case study (SHQ)
124	human faeces	Feb 1996 case study (SHQ)
125	human faeces	Feb 1996 case study (SHQ)
126	human faeces	Feb 1996 case study (SHQ)
127	human faeces	Feb 1996 case study (SHQ)
128	human faeces	Feb 1996 case study (SHQ)
129	human faeces	Feb 1996 case study (SHQ)

Key

YO - year old

Qld - Queensland

Vic - Victoria

NSW - New South Wales

Tas - Tasmania

NT - Northern Territory

SA - South Australia

WA - Western Australia

Table 2.2 - Details of other *Salmonella* and non - *Salmonella* strains

Strain	Source
Escherichia coli ACM 1803	Australian Collection of Microorganisms
Escherichia coli ATCC 25922 (ACM 1901)	Australian Collection of Microorganisms
Salmonella typhimurium ACM 3598	Australian Collection of Microorganisms
Salmonella typhimurium 82/6915	Royal Melbourne Institute of Technology - Victorian poultry isolate
Salmonella sofia Bt6	Royal Melbourne Institute of Technology - Victorian poultry isolate
Salmonella enteritidis SE6/E5 Phage type 13a	Provided by Dr JM Cox
Salmonella enteritidis ACM 3696 Phage type 4	Institute of Medical and Veterinary Science
Salmonella enteritidis ACM 3702 Phage type 7	Institute of Medical and Veterinary Science

While strains were not isolated during this study it was intermittently necessary to confirm strains and this was achieved using a *Salmonella* - specific latex agglutination test (Serobact Salmonella - Medvet Science Pty Ltd Australia) and a group C_1 specific agglutinating serum (Catalogue No. CZ15, Murex Diagnostics Ltd England).

Isolates were maintained as slope cultures on Tryptone Soy Agar (TSA) and stored at room temperature, in the dark. Freeze dried preparations and 15% glycerol stocks of all the strains listed in Table 2.1 were also prepared for long term storage.

2.2. b) Antibiotic susceptibility testing

Antimicrobial susceptibility testing of isolates 1-81 (see Table 2.1) was performed by the disk diffusion method as described in the Manual for Clinical Microbiology (Balows & Hausler, 1991). *E. coli* ATCC 25922 was used as the media control strain. Mueller-Hinton agar (Cat. No. CM 337, Oxoid Pty Ltd) certified for use in antimicrobial testing was used and the following six antimicrobial agents (Oxoid Pty Ltd) were tested using the disks indicated; ampicillin (10μg), chloramphenicol (30μg), ciprofloxacin (5μg), gentamicin (10μg), tetracycline (30μg) and trimethoprim/ sulphamethoxazole (25μg).

The zone of inhibition was measured (diameter in mm) and translated to resistant, intermediate, moderately susceptible and susceptible using the criteria from the National Committee for Clinical Laboratory Standards (NCCLS) Approved Standard M2-A5 (1993).

Furazolidone susceptibility was also tested for isolates 1-81. There are no standards for disk diffusion testing of furazolidone in the NCCLS standards of 1991 or 1993, therefore the minimum inhibitory concentrations (MIC) were determined by the agar dilution method, previously described by Rampling *et al.* (1990). Iso-sensitest Agar (Oxoid Australia Pty Ltd, CM 471) was used and doubling dilutions from 1mg/l to 256mg/l were tested. 10μl drops containing 10^4 cfu of *S. virchow* were used to inoculate the plates. This inoculum was prepared by diluting an overnight Iso-sensitest broth culture in sterile saline. After the drop of inoculum was allowed to dry the plates were incubated at 37°C for 16-18 hours. An MIC for furazolidone of ≥8mg/l was reported as resistant, MIC of 4mg/l was intermediate and an MIC of 2mg/l was considered susceptible (Cox *et al.*, 1996a).

2.2. c) Phage typing

Seventy nine isolates of *S. virchow* were phage typed (refer to Table 2.4) by the *Salmonella* Reference Laboratory, Laboratory of Enteric Pathogens, Public

Health Laboratory Service, Colindale, London, according to the method of Chambers *et al.* (1987).

2.2. d) DNA isolation techniques

Several techniques were used to isolate low molecular weight plasmid DNA including: rapid alkaline extraction (Birnbiom & Doly, 1979), a modification of alkaline extraction (Sambrook *et al.*, 1989), the rapid method of Kado and Lui (1981) and by boiling (Ausubel, 1987).

The method chosen as the most reliable and acceptable for screening large numbers of isolates, was the Wizard™ Minipreps DNA Purification System (Promega Corporation). The procedure was modified from the manufacturer's instructions because of apparent loss of *S. virchow* plasmid DNA due to endogenous nuclease activity. The modified method which maximized low molecular weight plasmid DNA yield was :-

1. Isolates were inoculated into a 20ml Luria-Bertani (LB) broth culture and incubated for 22 hours at 37°C without shaking
2. 10ml of a 22 hour culture was centrifuged at 4 350g for 5 minutes and the pellet was resuspended in 300µl of cell resuspension solution
3. 300µl of cell lysis solution was added and mixed gently by inversion
4. 300µl of neutralization solution was added and after inversion the tubes were left at room temperature for 10 minutes to efficiently precipitate proteins (including nucleases)
5. tubes were centrifuged at 17 320*g* in a microcentrifuge for 10 minutes and then the supernatant was transferred to a new tube
6. 1ml of purification resin was added and mixed by inversion
7. the resin/DNA mix was pushed through a minicolumn
8. the minicolumn was then washed with 2ml of column wash solution
9. the minicolumn was spun at 17 320*g* for 2 minutes to dry the resin and
10. 50µl of sterile dH_2O at room temperature was used to elute plasmid DNA by centrifugation at 17 320*g* for 20 seconds.

Following the column purification, phenol and phenol/chloroform treatments were included in order to obtain DNA of suitable quality to digest with restriction enzymes. The protocol for phenol treatment and subsequent ethanol precipitation of the plasmid DNA was :-

1. To a tube containing DNA eluted from the minicolumn, an equal volume of phenol (unbuffered) (Sigma-Aldrich Pty Ltd) was added and then vortexed for 15 seconds
2. After 5 minutes at room temperature the tube was centrifuged at 17 320*g* in a microcentrifuge for 3 minutes
3. The aqueous layer was transferred to a fresh tube and an equal volume of phenol/chloroform (1:1, unbuffered) was added and vortexed for 15 seconds
4. The tube was centrifuged at 17 320*g* for 3 minutes and the aqueous layer was again transferred to a fresh tube
5. Two volumes of cold (-20°C) 100% ethanol was added and mixed well by inversion
6. Tubes were stood on ice for 5 minutes and then centrifuged at 4°C for 5 minutes
7. 100% ethanol was decanted and 0.5ml of 70% ethanol (4°C) was added and the tube was then centrifuged at 4°C for 3 minutes
8. The 70% ethanol was decanted and the pellet was dried using a Speed Vac concentrator (Savant Instruments Inc.)
9. Once dry, the DNA was resuspend in 50µl of sdH_2O by vortexing gently and the plasmid DNA was stored at -20°C.

Two methods were trialed for the preparation of genomic DNA for ribotyping and IS*200* typing. The first method was the method of Ausubel *et al.* (1987) but the method chosen for routine use was the method of Christensen *et al.* (1993). This was the preferred method because it was simple and resulted in consistent amounts of genomic DNA that was readily digestable by restriction enzymes.

Preparations of both plasmid DNA and genomic DNA were quantified using a spectrophotometer and the absorbance at 260nm, knowing that dsDNA at 50ug/ml has an A_{260} of 1.

2.2. e) Restriction digestion and agarose gel electrophoresis

2.2. e (i) Plasmid restriction endonuclease digests

The low molecular weight plasmid DNA of various *S. virchow* isolates were digested with the restriction enzymes, *Hae*III and *Alu*I (New England BioLabs Inc). Digests were set up in 20µl volumes as follows :

plasmid DNA.....variable
10x buffer 2µl
*Hae*III/*Alu*I enzyme 10U/8U
sterile dH_2O .....to total 20µl

Digests were incubated for 1 hour at 37°C and the enzymes were heat inactivated at 65°C for ten minutes.

2.2. e (ii) Genomic digests for ribotyping

A selection of enzymes were trialed to determine those which resulted in the most distinct ribotyping patterns. *Pst*I, *Hin*dIII, *Ban*I and *Sma*I (New England BioLabs Inc.) were used to digest *S. virchow* genomic DNA. Only *Ban*I generated a discriminatory banding profile and was therefore chosen for routine use with all isolates. Digests were performed in 15µl volumes as follows :

genomic DNA..... 10µg
10x buffer 1.5µl
*Ban*I enzyme..... 40U
sterile dH_2O 6.5µl

Digests were incubated for 3 hours at 37°C and heat inactivated at 65°C for 10 minutes.

2.2. e (iii) Genomic digests for IS*200* typing

*Ban*I, *Pst*I, *Bgl*II and *Pvu*II were used to digest *S. virchow* genomic DNA to determine which was the most appropriate for IS*200* typing. It was *Ban*I again which gave the most easily interpreted patterns and the most diverse number of bands which hybridized to the probe. Digests were performed as outlined in 2.2e ii.

2.2. e (iii) Agarose gel electrophoresis of nucleic acids

Electrophoresis of low molecular weight plasmid DNA was performed using 0.7% agarose (Type V, Sigma-Aldrich Pty Ltd) gels electrophoresed in 1 x Tris Borate EDTA (TBE) buffer (pH 8) (Sambrook *et al.*, 1989) at 6.5V/cm (100V) for approximately 30 minutes. Ethidium bromide was incorporated into both the chamber buffer and the gel at a concentration of 0.5 - 1μg/ml.

Electrophoresis of genomic DNA digested in preparation for ribotyping or IS*200* typing, was performed using 0.8% agarose (Type V, Sigma-Aldrich Pty Ltd) gels electrophoresed in 1 x Tris Acetate EDTA (TAE) buffer (pH 8.5) (Sambrook *et al.*, 1989) at 1V/cm (30V) for 12-14 hours. Ethidium bromide was incorporated into the buffer chamber and gels at a concentration of 1μg/ml.

2.2. f) Ribotyping and IS*200* typing methodology

The following methodology was applied for both ribotyping and IS*200* typing except where any specific differences are outlined.

Genomic DNA was digested with an appropriate enzyme (section 2.2e ii) and the restricted DNA and a DIG-labelled molecular weight marker (DIG-labelled molecular weight marker II, Catalogue No. 1218590 - Boehringer Mannheim Australia Pty Ltd) were electrophoresed (section 2.2e iii). After electrophoresis, the DNA was transferred from the agarose gel to Hybond N nylon hybridization membrane (Amersham International plc) using the method of Southern (1975), employing vacuum transfer with the Vacu-Gene apparatus (Pharmacia (Australia) Pty Ltd). The reagents, times and strength of vacuum used were as follows :

1. Depurination ..... 0.2N HCl ..... 50mbar ..... 4 minutes
2. Denaturation..... 0.5N NaOH / 1.5M NaCl..... 40mbar ..... 3 minutes
3. Neutralization..... 1.5M NaCl / 1M Tris..... 40mbar ..... 3 minutes
4. Transfer..... 20 X SSC..... 40mbar ..... 55 minutes

DNA was cross-linked to the membrane by exposure to UV radiation for 4 minutes per side of membrane. Membranes were stored at 4°C wrapped in aluminium foil until hybridization.

All probes were labelled by the incorporation of alkaline-labile digoxigenin-11-dUTP by PCR using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim Australia Pty Ltd). The reaction conditions and cycling program used are described below. For ribotyping, the probe was prepared by PCR DIG labelling of genomic DNA from *E. coli* ACM 1803. The primers used were the two-fold degenerate 16S ribosomal RNA (rRNA) sequencing primers, 27 forward (27f) and 1492 reverse (1492r) (Lane, 1991) (primer sequences below). The DIG-labelled probe for IS*200* typing was prepared using genomic DNA extracted from *S. typhimurium* ACM 3598 and this DNA was added either directly to the PCR reaction or after pre-digestion with the restriction endonuclease *Pst*I. *Pst*I does not cleave within the insertion sequence and this step has been reported to increases the yield of PCR product by making the target sequences more accessible to the primers (Baquar *et al.*, 1993). The primers used for IS*200* probe synthesis were those published by Baquar *et al.* (1993) (primer sequences below).

The primer sequences were :

27f 5' AGAGTTTGATCMTGGCTCAG 3' *

1492r 5' TACGGYTACCTTGTTACGACTT 3' *

* degenerate code; M = C:A, Y = C:T

IS*200*f 5' ATAGCCGAGGTTTTTCAGATGC 3'

IS*200*r 5' AGTCTATGGAAACCCCCAGC 3'

The PCR reactions were set up in 50µl volumes as follows :-

PCR buffer without $MgCl_2$ (10x)..... 5µl
$MgCl_2$ (25mM stock)..... 3µl
PCR DIG mix (10 x)..... 5µl
forward primer*..... 5µl
reverse primer*..... 5µl
Taq DNA polymerase (5.5U/µl)..... 0.1µl
Template DNA 100pg-1µg
sterile dH_2O ..... variable to total 50µl

* The concentration of the IS*200* primers was 40ng/µl and the ribotyping primers were at a concentration of 200ng/µl.

The thermal cycling conditions for the PCR labelling reaction were :-

96°C 4 minutes
↓
30 cycles of :
95°C 45 seconds
60°C 1 minute
72°C 2 minutes
↓
4°C soak

After PCR a 5µl aliquot of the reaction was electrophoresed on a 0.7% agarose gel containing ethidium bromide to visualize the digoxigenin labelled product. The sizes of the PCR products were determined by comparison with molecular weight markers. However, it should be noted that incorporation of the DIG moiety into the labelled product renders it slightly larger than the expected size of 1500-1600bp for the ribotyping probe and 692bp for the IS*200* probe. After confirmation that a DIG labelled PCR product was obtained it was further purified. The ribotyping probe was

purified using the Magic™ PCR Preps DNA Purification System (Promega Corporation) and the IS*200* probe was purified using an agarose gel DNA extraction kit (Boehringer Mannheim Australia Pty Ltd). Both kits were used as per the manufacturer's instructions. Lastly, an aliquot of the labelled probe was spotted onto nylon membrane and a direct detection was performed to quantitate the amount of labelled probe. The probe was not divided into aliquots for single use in each hybridization but rather all of the prepared probe was used in the first hybridization, recovered after hybridization and reused several times.

The hybridizations and detections were performed using the Digoxigenin Non-Radioactive DNA labelling and detection kit (Boehringer Mannheim) as per the manufacturer's instructions. Hybridization was performed at 68°C for the ribotyping probe and at both 68°C and 42°C for the IS*200* probe. Hybridizations were incubated overnight and the first stringency washes were at room temperature and the second washes were at 68°C. Colourimetric detection using anti-digoxigenin Fab fragments conjugated to alkaline phosphatase and substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP - X-Phosphate) were employed. Detection was allowed to progress, usually overnight and the banding patterns were visually compared. Each isolate was tested at least twice to confirm their pattern.

2.2. g) IS*200* PCR protocol

To confirm whether the *S. virchow* isolates under investigation possessed IS*200*, a representative group of isolates were subjected to touchdown PCR, using the IS*200*f and IS*200*r primers. The *S. virchow* isolates tested were 22, 29, 33, 34, 44, 50, 54, 56, 76, 87, 91, 93, 99 and 100. *S. typhimurium* ACM 3598 was included as a positive control because it is known to have between 6 and 10 copies of the IS*200,* depending on the particular strain (Lam & Roth, 1983). The PCR was optimized for *S. typhimurium* ACM 3598 DNA and the parameters were adopted for the *S. virchow* screening. The standardized parameters were 1µg of template DNA and 2mM $MgCl_2$.

The IS*200* PCR reactions were set up as follows :

PCR buffer without $MgCl_2$ (10x)..... 5µl
$MgCl_2$ (25mM stock)..... 4µl
dNTPs (1.25mM)..... 8µl
forward primer (IS*200*f) (40ng/µl)..... 5µl
reverse primer (IS*200*r) (40ng/µl)..... 5µl
Taq DNA polymerase (5.5U/µl)..... 0.2µl
Template DNA 1µg
sterile dH_2O ..... variable to total 50µl

The cycling conditions for the touchdown PCR were :

95°C 4 minutes
↓
2 cycles each of :
95°C, 45 seconds, → 60°C, 1 minute, → 72°C, 2 minutes
95°C, 45 seconds, → 58°C, 1 minute, → 72°C, 2 minutes
95°C, 45 seconds, → 55°C, 1 minute, → 72°C, 2 minutes
95°C, 45 seconds, → 52°C, 1 minute, → 72°C, 2 minutes
↓
30 cycles of :
95°C, 45 seconds, → 50°C, 1 minute, → 72°C, 2 minutes
↓
1 cycle of :
95°C, 45 seconds, → 48°C, 1 minute, → 72°C, 5 minutes
↓
4°C soak

2.2. h) Cloning and sequencing of the IS*200* of *S. typhimurium*

2.2. h (i) TA cloning of the IS*200* PCR product

The IS*200* PCR product of *S typhimurium* ACM 3598 was cloned using the pGEM®-T vector system (Promega Corp.) as per the manufacturer's instructions. The PCR product was first gel purified from a 1% agarose gel using the agarose gel DNA extraction kit (Boehringer Mannheim Australia Pty Ltd). The purified DNA was eluted in 70µl of 1mM Tris-HCl pH 8 at room temperature. The ligation of purified PCR product and pGEM®-T vector was performed at a nanogram ratio of 3:1 (PCR product:vector) and set up as per the instructions with the addition of 1µl of fresh 10mM ATP to each ligation mixture. The ligation reaction was incubated overnight beginning at room temperature and decreasing to 4°C.

After overnight incubation, electroporation of the ligated vector was performed using 1.5µl of the ligation mix and 40µl of electrocompetent *E. coli* DH5α cells (these cells were kindly provided by Craig Belcher and were prepared in salt free nutrient yeast extract broth). Electroporation was done at 1.8kilovolts, 25µFaraday and 200Ω. Immediately after electroporation 1ml of SOB media was added to the cells (Sambrook *et al.*, 1989) and the resulting suspension was incubated at 37°C for one hour without shaking. Aliquots ranging from 20 to 200µl were plated onto Luria-Bertani (LB) agar containing 100µg/ml of ampicillin, 80µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) and 80µg/ml of isopropyl-β-D-thiogalactopyranoside (IPTG). The plates were incubated for 16-18 hours at 37°C to allow blue/white selection of colonies containing vector and insert.

Using the blue/white selection twelve of the white colonies (containing inserts) were chosen and inoculated into LB broth containing 100µg/ml ampicillin and the recombinant plasmid DNA was isolated using the miniprep procedure from Sambrook *et al.* (1989). The plasmid DNA from these twelve colonies was *Apa*I/*Spe*I double restriction digested and agarose gel electrophoresed to confirm the presence of cloned IS*200* DNA.

The double digests were set up as follows in 20µl reaction volumes and incubated at 37°C for 2 hours:-

miniprep DNA 3ul
10x buffer 2ul
*Apa*I 10U
*Spe*I 7.5U
sterile dH_2O 14ul

One of the plasmid preparations was chosen to perform a subcloning. The remainder of this plasmid preparations and the others were stored at -20°C.

2.2. h (ii) Subcloning and sequencing of the IS*200* PCR product

Subcloning of the ca. 700bp *IS200* DNA from plasmid construct pGEM®-T IS*200* was performed using the asymmetric *Eco*RI restriction site within IS*200*.

6ug of the cloned IS*200* DNA was digested from the pGEM®-T vector using two double digests, *Apa*I/*Eco*RI and *Spe*I/*Eco*RI. 1ug of the subcloning vector pBluescript (Stratagene Cloning Systems) was also prepared by similar double digestion for 2 hours at 37°C. After digestion the reactions were electrophoresed on 0.7% agarose gels. The 420bp and 290bp fragments of IS*200* and the digested pBluescript were excised from the gel and purified using QIAquick™ (QIAGEN GmbH) following the manufacturer's instructions and finally eluted in 150µl of water each.

Both IS*200* DNA fragments were co-precipitated with the appropriately double digested vector at a nanogram ratio of 2:1 (DNA:vector) using 3M sodium acetate pH5.2 and 2.5 volumes of cold 100% ethanol, held at -70°C for several hours and pelleted at 17 320*g* at 4°C for 20 minutes. The pellet was washed with 70% ethanol and dried using a Speed Vac concentrator (Savant Instruments Inc.).

The DNA pellet was resuspended in 2ul of sterile dH_2O and incubated for five minutes at 55°C. 2ul of double strength ligation mix containing 2mM ATP, 2 x ligation buffer, 1U of T4 ligase and sterile dH_2O was added and ligation allowed to proceed overnight beginning at room temperature and decreasing to 4°C.

Electroporation was performed and plated on LB agar containing ampicillin, X-Gal and IPTG as outlined in 2.2h i.

White colonies were obtained from both subclonings and plasmid DNA was extracted by the modified alkaline lysis / PEG precipitation method as recommended by the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit protocol - part number 401388 (Applied Biosystems, Inc.). Double digests were performed on the plasmid preparations to ensure insert DNA of the expected size was present. A representative *Spe*I/*Eco*RI subclone was chosen and sequenced according to the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit protocol using pUC M13f and pUC M13r 17-mer primers (Promega Corp). The *Apa*I/*Eco*RI subcloning was not successful and so to obtain sequence data for the entire IS*200* sequence the miniprep extracted pGEM®-T cloned complete IS*200* DNA was PEG precipitated and sequenced as for the *Spe*I/*Eco*RI subclone.

The extension products were extracted using phenol/chloroform and ethanol precipitated as outlined in the Applied Biosystems protocol. The sequencing gels were performed by the DNA Sequencing Facility, University of Queensland. The results of the automated sequencing were compiled using SeqEd (Applied Biosystems, Ca., USA). The analysis and multiple alignments were done using BlastN 1.4.9MP (National Center for Biotechnology Information (NCBI) Blast Network Service) (Altschul *et al.*, 1990) and Clustal W (1.5) multiple sequence alignment tool through the Australian National Genome Information Service (ANGIS).

2.2. i) Application of typing methods to outbreak isolates

A group of nine *S. virchow* isolates were obtained from State Health, Queensland that were known to have originated from faecal samples of individuals involved in a food poisoning incident. Ribotyping and plasmid profiling were applied to this group of known epidemiologically - related isolates as outlined in Sections 2.2d and 2.2f. The isolates were obtained too late in the project to allow them to be phage typed by PHLS in London.

2.3 Results

This section contains the results of the five typing methods which were performed on the *S. virchow* isolates and where appropriate, other bacterial strains which were used as controls or for comparison. The results of a small case study using a group of nine *S. virchow* isolates (isolates 121-129) from a food-borne outbreak are also presented.

2.3. a) Antimicrobial susceptibility testing

Seventy-four *S. virchow* isolates were tested against six antimicrobial agents using the disk diffusion method and against furazolidone using the agar dilution method (refer to Section 2.2 b). The diameters of the zones of inhibition for the disk diffusion test were converted to resistant, intermediate or susceptible using the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (1993). Isolates 1-3, 5-34, 39, 40, 42-47, 73, 75, 76, 79-81 were susceptible to all of the antimicrobial agents tested.

The results for minimum inhibitory concentration (MIC) determination against furazolidone were recorded as sensitive or resistant using the MIC limits defined by Cox *et al.* (1996) (refer to section 2.2 b). All isolates tested, except 4, 55 and 56, were sensitive to furazolidone with MICs of 1 or 2 μg/ml. Isolates 4, 55 and 56 were resistant with MICs of 16μg/ml.

Table 2.3 presents the antimicrobial resistance patterns for isolates which exhibited resistances (including intermediate resistance) to one or more of the antimicrobial agents. Figure 2.1 is a photograph of the furazolidone susceptibility plates showing the MIC determination of isolates 55 and 56.

Table 2.3 - Disk Diffusion and agar dilution susceptibility results

Isolate	Gm 10µg	Cm 30µg	Tm/Sx 25µg	Tet 30µg	Amp 10µg	Cipro 5µg	Fz MIC
4	S	S	S	S	S	S	R
35	I	S	S	R	R	S	S
36	I	S	S	R	S	S	S
37	S	S	S	R	S	S	S
38	R	S	S	R	I	S	S
41	S	S	S	I	S	S	S
48	S	S	S	I	S	S	S
50	S	S	S	R	I	I	S
51	R	I	S	R	S	S	S
52	S	I	S	I	S	S	S
53	S	I	S	R	R	S	S
54	S	S	R	I	R	S	S
55	I	R	S	S	R	S	R
56	S	S	S	I	S	S	R
58	S	S	S	I	R	S	S
59	S	S	S	I	R	S	S
60	I	S	S	I	R	S	S
61	S	S	S	R	R	S	S
62	R	I	I	R	R	S	S
63	R	R	S	R	S	S	S
64	S	S	S	I	R	S	S
66	R	S	S	R	S	I	S
67	I	R	S	I	S	S	S
68	R	R	I	R	R	I	S
69	S	S	S	R	S	S	S
71	I	S	S	R	S	S	S
72	S	S	S	I	S	S	S

Isolate	Gm 10μg	Cm 30μg	Tm/Sx 25μg	Tet 30μg	Amp 10μg	Cipro 5μg	Fz MIC
74	S	S	S	I	S	S	S
77	S	S	S	I	S	S	S
78	S	S	S	I	S	S	S

Key

Amp - ampicillin

Cipro - ciprofloxacin

Cm - chloramphenicol

Fz - furazolidone

Gm - gentamicin

I - intermediate

MIC - minimum inhibitory concentration

R - resistant

S - susceptible

Tet - tetracycline

Tm/Sx - trimethoprim/sulphamethoxazole

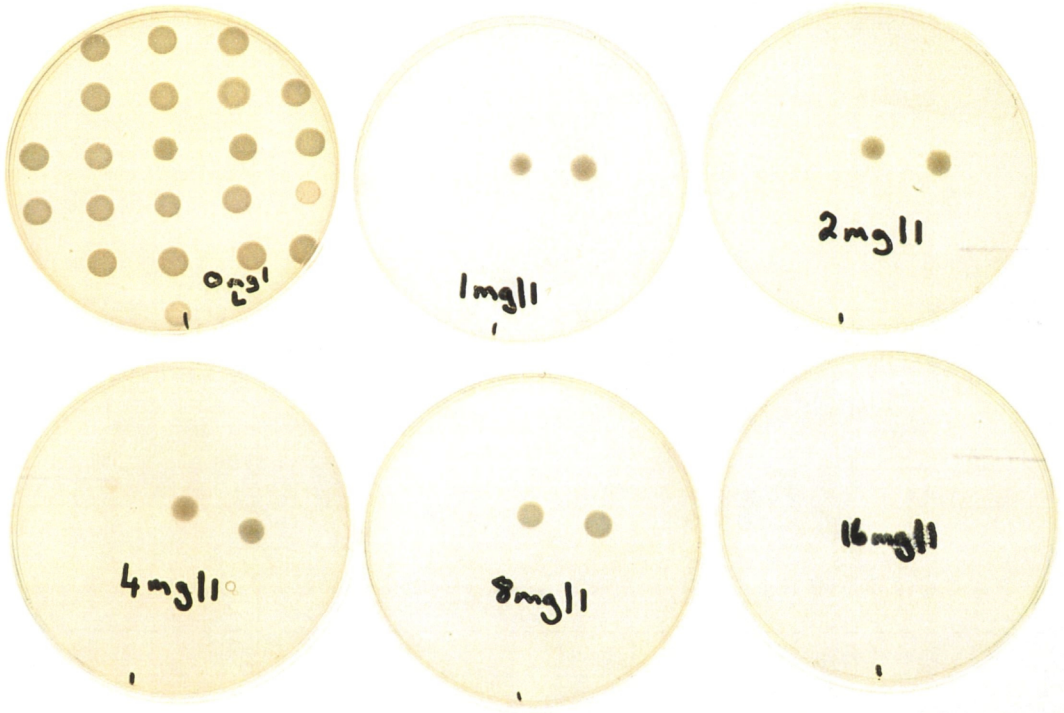


Figure 2.1- Furazolidone MIC determination plates. Iso-sensitest agar plates containing furazolidone at concentrations of 0, 1, 2, 4, 8 and 16 mg/l, spotted with 10^4 colony forming units (cfu) of isolates 46-69. Isolates 55 and 56 show resistance to 8mg/l

2.3. b) Phage typing

Seventy - nine *S. virchow* isolates were phage typed by PHLS Colindale (refer to section 2.2 c). Seventy-six isolates were allocated to phage types which had been identified using the *S. virchow* scheme established by Chambers *et al.* (1987) and three isolates were RDNC (react but did not conform). RDNC means that the bacterial strain was susceptible to phages from the standard set but the pattern of susceptibility was not one of the recognized phage type patterns. Thirteen phage types including subtypes were represented. Phage type 8 was the most prevalent type representing fifty-four of the seventy-nine isolates (68.3%). Table 2.4 presents the results with the isolates grouped by their phage type.

Table 2.4 - Phage typing results of *S. virchow* isolates

Isolate	Phage type	Source
3	8	possum
4	8	human
5	8	macadamia nuts
7	8	human
9	8	chicken meat
11	8	chicken meat
12	8	chicken meat
13	8	chicken meat
14	8	chicken meat
15	8	chicken meat
16	8	chicken meat
17	8	chicken meat
18	8	chicken meat
19	8	chicken meat
20	8	chicken meat
22	8	chicken meat
23	8	chicken meat
24	8	chicken meat
25	8	chicken meat
26	8	chicken meat
27	8	chicken meat
29	8	chicken meat
30	8	chicken meat
31	8	human
35	8	human faeces
36	8	human faeces
37	8	human faeces
39	8	human faeces
40	8	human faeces
42	8	chicken meat
46	8	sewage effluent Sydney
52	8	human faeces
53	8	human faeces
54	8	human faeces
55	8	human faeces
61	8	wallaby lymph node
63	8	wallaby liver
68	8	porcine pus
69	8	cane toad faeces
71	8	human
73	8	human faeces
74	8	human faeces
76	8	chicken meat

Isolate	Phage type	Source
77	8	human faeces
78	8	chicken meat
79	8	human faeces
80	8	chicken meat
81	8	human faeces
88	8	human faeces
90	8	human faeces
92	8	human blood
96	8	human faeces
97	8	human hip-plate
99	8	human faeces
82	8a	human faeces
38	34	human faeces QLD
51	34	human faeces QLD
60	34	bovine faeces QLD
64	34	kangaroo faeces QLD
72	34	human faeces QLD
89	34a	human faeces QLD
91	34a	human faeces QLD
1	31	meat/bone meal
2	31	frog's legs
34	31	soya bean - feed

Isolate	Phage type	Source
43	36	sewage effluent Penrith
93	36	human faeces QLD
94	36(variant)	human faeces QLD
45	RDNC	sewage effluent Penrith
67	RDNC	bovine duodenum QLD
87	RDNC	human faeces VIC (travel Africa)
83	11	human faeces VIC
85	11	human faeces VIC
47	15	chicken faeces QLD
50	15	human faeces QLD
84	7a	human faeces TAS (travel Fiji)
41	23	chicken meat QLD
95	24	human faeces QLD
101	25	human faeces QLD

RDNC - react but does not conform

2.3. c) Plasmid profiling

Ninety-five *S. virchow* isolates were examined for the presence of low molecular weight plasmids (refer to Section 2.2 d). Five different plasmid profiles were observed and assigned as PP 1 to PP 5. Plasmid profile 6 consisted of the isolates which had no low molecular weight plasmids. Table 2.5 shows the isolates with plasmid profiles 1-5.

Twenty-two of the ninety-five isolates (34%) contained plasmid DNA of low molecular weight. Each of these isolates possessed only one plasmid. Electrophoresis of the plasmid DNA on agarose gels and staining with ethidium bromide generally allowed visualization of the three forms of plasmid (open circular, nicked and closed circular). Figure 2.2 is a photograph of typical plasmid DNA isolated.

Table 2.5 - Plasmid profiles using low molecular weight plasmids

Isolate	Plasmid Profile	Source
8	1	chicken meat
15	1	chicken meat
23	1	chicken meat
24	1	chicken meat
25	1	chicken meat
26	1	chicken meat
27	1	chicken meat
28	1	chicken meat
29	1	chicken meat
30	1	chicken meat
41	1	chicken meat QLD
75	1	chicken liver/heart
76	1	chicken meat
78	1	chicken meat
80	1	chicken meat
43	2	sewage effluent Penrith
44	2	sewage effluent Penrith
45	2	sewage effluent Penrith
42	3	chicken meat
86	3	human faeces
52	4	human faeces
82	5	human faeces

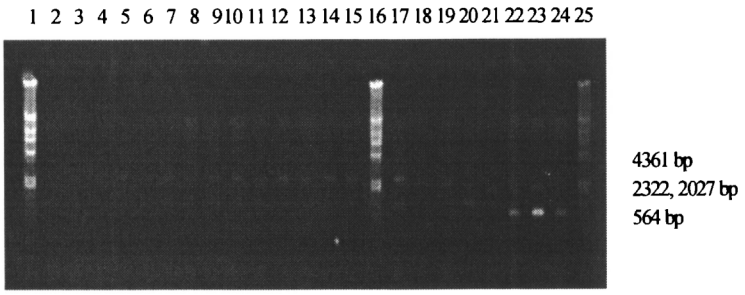


Figure 2.2 - Ethidium bromide stained agarose gel of plasmid DNA from all isolates containing low molecular weight plasmids. Lane 1, 16, 25 - *Hind*III digested Lambda DNA marker, lane 2 - isol. 8, lane 3 - isol. 75, lane 4 - isol. 76, lane 5 - isol. 78, lane 6 - isol. 80, lane 7 - isol. 41, lane 8 - isol. 23, lane 9 - isol. 24, lane 10 - isol. 25, lane 11 - isol. 26, lane 12 - isol. 27, lane 13 - isol. 28, lane 14 - isol. 29, lane 15 - isol. 30, lane 17 - isol. 15, lane 18 - isol. 86, lane 19 - isol. 42, lane 20 - isol. 52, lane 21 - isol. 82, lane 22 - isol. 43, lane 23 - isol. 44, lane 24 - isol. 45

To ensure that all isolates within each plasmid profile possessed the same plasmid, restriction digests were performed to generate a specific plasmid fingerprint (refer to section 2.2e i). Plasmid DNA was re-extracted for digestion but the plasmid DNA could not be re-extracted from five of the isolates (15, 41, 42, 52, 86). All of the isolates in the study had been subcultured several times in the intervening period and it was possible that the plasmids were lost during this time because no selective pressure was applied during subculturing and storage.

Plasmid DNA from thirteen of the fifteen isolates with plasmid profile 1 were digested with the restriction enzyme *Hae*III (refer to Section 2.2e i). The same restriction pattern or plasmid fingerprint was observed for all of the plasmids. The fragments produced included one of approximately 1600bp and several unresolved bands of less than 750bp (Figure 2.3). *Hae*III digestion of plasmid DNA from plasmid profile 2 was also performed and Figure 2.4 shows that all three members of profile 2 showed an identical fingerprint. The plasmid fingerprints generated by the plasmid DNA of PP 1 and 2 were different from each other.

Plasmid DNA from members of plasmid profile 2 were also digested with the restriction enzyme *Alu*I and again all members of the group showed the same pattern which consisted of approximately seven bands all less than 1000bp (data not shown).

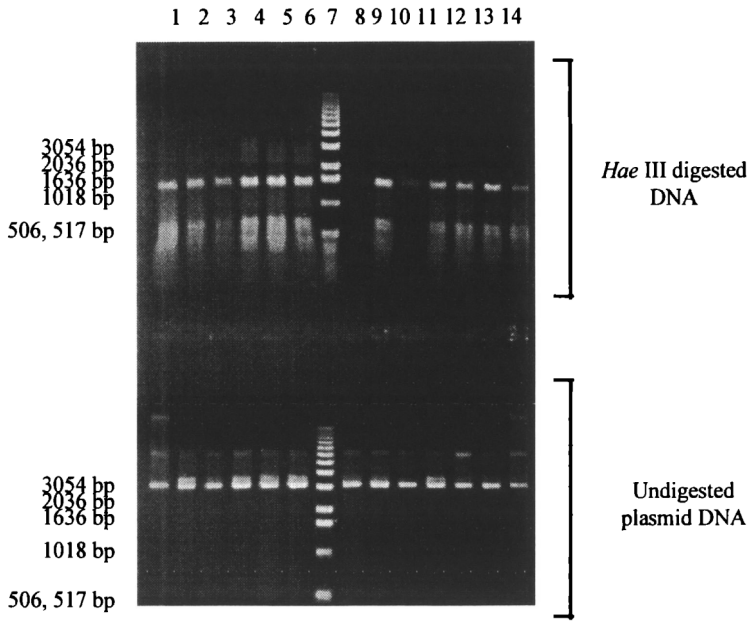


Figure 2.3 - Undigested and *Hae*III digested plasmid profile 1 DNA. Lane 1 - isol. 8, lane 2 - isol. 75, lane 3 - isol. 78, lane 4 - isol. 80, lane 5 - isol. 76, lane 6 - isol. 23, lane 7 - 1 Kb ladder, lane 8 - isol. 24, lane 9 - isol. 25, lane 10 - isol. 26, lane 11 - isol. 27, lane 12 - isol. 28, lane 13 - isol. 29, lane 14 - isol. 30

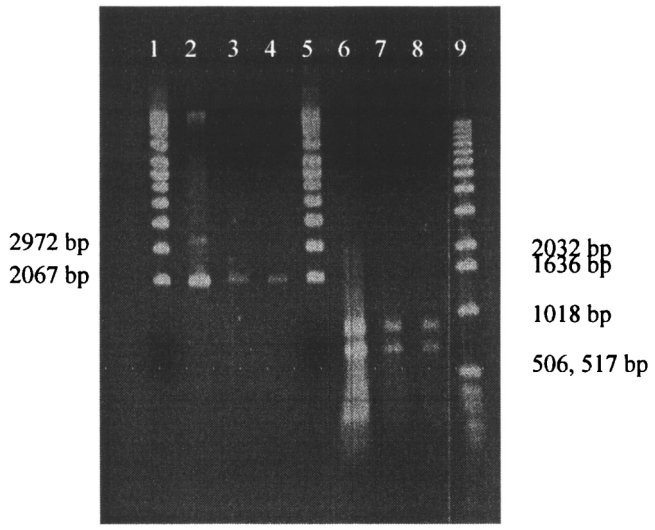


Figure 2.4 - Undigested and *Hae*III digested plasmid profile 2 DNA. Lane 1 and 5 - Supercoiled DNA ladder, lane 9 - 1 Kb Ladder DNA , lane 2 - undigested isol. 43, lane 3 - undigested isol. 44, lane 4 - undigested isol. 45, lane 6 - digested isol. 43, lane 7 - digested isol. 44, lane 8 - digested isol. 45

2.3. d) Ribotyping

Ninety-four *S. virchow* isolates, *S. sofia* Bt6 and *S. typhimurium* ACM 3598 were ribotyped (refer to Section 2.2f). To provide a consistency of recording between blots only bands with a molecular weight of less than 6557bp were used in the analysis. There were eight ribotypes identified and the most common pattern (87%) was designated Ribotype 1 (RT1). Table 2.6 provides the results with isolates grouped according to their ribotypes. Figure 2.5 is an example of a ribotyping blot showing six of the eight ribotypes.

Table 2.6 - Results of ribotyping using *E. coli* 16s rRNA probe

Isolate	Ribotype	Source
67	1	bovine duodenum QLD
60	1	bovine faeces QLD
69	1	cane toad faeces
47	1	chicken faeces
48	1	chicken faeces
75	1	chicken liver/heart
8	1	chicken meat
9	1	chicken meat
11	1	chicken meat
13	1	chicken meat
14	1	chicken meat
15	1	chicken meat
16	1	chicken meat
17	1	chicken meat
18	1	chicken meat
19	1	chicken meat
20	1	chicken meat
21	1	chicken meat
22	1	chicken meat
23	1	chicken meat
24	1	chicken meat
25	1	chicken meat
26	1	chicken meat
27	1	chicken meat
28	1	chicken meat
29	1	chicken meat
30	1	chicken meat
41	1	chicken meat
42	1	chicken meat
76	1	chicken meat
78	1	chicken meat
80	1	chicken meat
7	1	human
31	1	human
71	1	human
92	1	human blood
35	1	human faeces
36	1	human faeces
37	1	human faeces
38	1	human faeces
39	1	human faeces
40	1	human faeces
50	1	human faeces
51	1	human faeces
52	1	human faeces
53	1	human faeces
54	1	human faeces
59	1	human faeces
72	1	human faeces
73	1	human faeces
74	1	human faeces

Isolate	Ribotype	Source
77	1	human faeces
79	1	human faeces
81	1	human faeces
82	1	human faeces
83	1	human faeces
85	1	human faeces
86	1	human faeces
88	1	human faeces
89	1	human faeces
90	1	human faeces
91	1	human faeces
93	1	human faeces
94	1	human faeces
95	1	human faeces
96	1	human faeces
99	1	human faeces
101	1	human faeces
97	1	human hip-plate
57	1	human urine
64	1	kangaroo faeces
66	1	kangaroo faeces
5	1	macadamia nuts
68	1	porcine pus
3	1	possum
43	1	sewage effluent Penrith
44	1	sewage effluent Penrith
45	1	sewage effluent Penrith
46	1	sewage effluent Sydney
63	1	wallaby liver
61	1	wallaby lymph node
62	1	wallaby lymph node

Isolate	Ribotype	Source
2	2	frog's legs
1	2	meat/bone meal
34	2	soya bean - feed QLD
4	3	human acquired o/s
55	3	human faeces
84	3	human faeces TAS (travel Fiji)
56	4	human faeces
100	4	human faeces
58	4	seawater
33	5	chicken faeces
87	6	human faeces VIC (travel Africa)
98	7	sheep faeces
121	8	human faeces outbreak
122	8	human faeces outbreak
123	8	human faeces outbreak
124	8	human faeces outbreak
125	8	human faeces outbreak
126	8	human faeces outbreak
127	8	human faeces outbreak
128	8	human faeces outbreak
129	8	human faeces outbreak

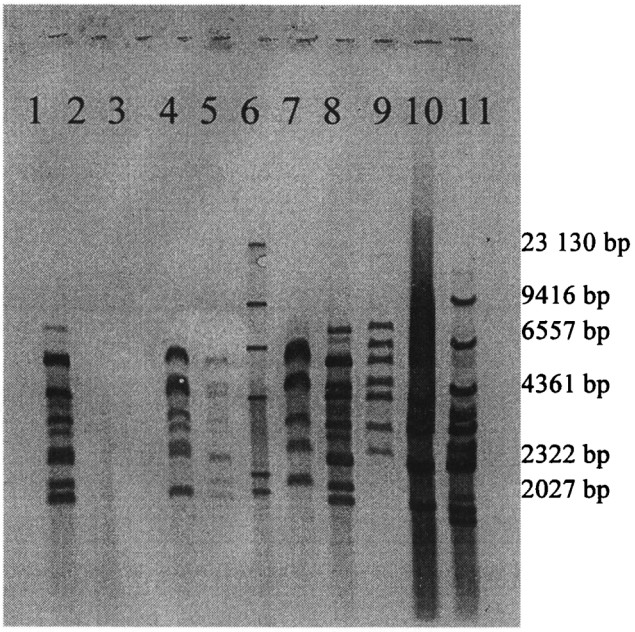


Figure 2.5 - Ribotyping blot of representatives of some of the ribotypes. Lane 1 - isol. 20 (RT1), lanes 2 and 3 - blank, lane 4 - isol. 56 (RT 4), lane 5 - isol. 58 (RT4), lane 6 - DIG labelled *Hind*III digested Lambda DNA marker, lane 7 - isol. 33 (RT5), lane 8 - isol. 87 (RT6), lane 9 - isol. 98 (RT7), lane 10 - isol. 125 (RT8), lane 11 - *S. typhimurium* ACM 3598

2.3. e) IS*200* typing

IS*200* typing was performed as outlined in Section 2.2f and results were obtained for 63 of the *S. virchow* isolates (1-11, 13-26, 60-101). IS*200* patterns were also obtained for *S. sofia* Bt6 and *S. typhimurium* ACM 3598. There were eight IS*200* types (IST) identified for *S. virchow* which arise due to differences in the copy number of the insertion sequence and the restriction profile of the genomic DNA containing these sequences. These differences result in different numbers and sizes of bands which hybridized to the probe in different isolates. The majority of *S. virchow* isolates (88.9%) belonged to IS*200* type 1 which had only one band. Table 2.7 shows the isolates grouped by IS*200* type and Figure 2.6 shows an example of an IS*200* blot.

Table 2.7 - Results of IS*200* typing

Isolate	IS*200* type	Source
3-5, 7-9, 13-26, 60-86, 88-97, 99, 101	1	various (see Table 2.1)
1	2	meat/bone meal
2	3	frog's legs
11	4	chicken meat
62	5	wallaby lymph node
87	6	human faeces VIC (travel Africa)
98	7	sheep faeces
100	8	human faeces

While performing IS*200* typing technical difficulties arose which resulted in no bands being detected for the positive control strain of *S. typhimurium* ACM 3598 or any of the *S. virchow* isolates. This included failure to detect bands for isolates which had already been tested and found to have hybridizing bands.

Genomic DNA from all of the isolates was re-extracted and digested. Gel electrophoresis of both the freshly extracted DNA and the frozen genomic DNA samples showed that both were being digested fully and were well separated by electrophoresis.

In order to test the transfer of digested DNA to nylon membranes, both old new Hybond N membrane was used. Transfer and hydridization solutions were prepared fresh before use. Results demonstrated that transfer was occurring satisfactorily, as indicated by the DIG-labelled *Hind*III digested λ DNA marker being transferred and detected. However this did not confirm successful hybridization as detection of the marker does not require probing, the DNA fragments of the marker are themselves labelled. At this stage no bands from the genomic digests appeared to bind the probe.

To test the sensitivity of detection control DNA provided in the Non Radioactive Nucleic Acid labelling and detection kit (Boehringer Mannheim Australia Pty Ltd) was spotted onto the membrane and detected. This established that both new and old detection reagents were satisfactory.

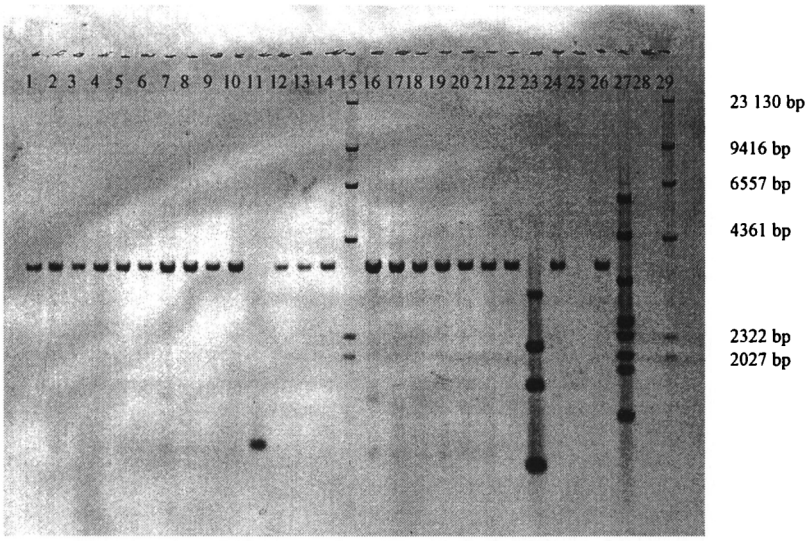


Figure 2.6 - IS200 typing blot of representatives of some of the IS200 types. Lane 15 and 29 - DIG labelled *Hind*III digested Lambda DNA marker, lane 1 - isol. 77 (IST1), lane 2 - isol. 78 (IST1), lane 3 - isol. 79 (IST1), lane 4 - isol. 80 (IST1), lane 5 - isol. 81 (IST1), lane 6 - isol. 82 (IST1), lane 7 - isol. 83 (IST1), lane 8 - isol. 84 (IST1), lane 9 - isol. 85 (IST1), lane 10 - isol. 86 (IST1), lane 11 - isol. 87 (IST6), lane 12 - isol. 88 (IST1), lane 13 - isol. 89 (IST1), lane 14 - isol. 90 (IST1), lane 16 - isol. 91 (IST1), lane 17 - isol. 92 (IST1), lane 18 - isol. 93 (IST1), lane 19 - isol. 94 (IST1), lane 20 - isol. 95 (IST1), lane 21 - isol. 96 (IST1), lane 22 - isol. 97 (IST1), lane 23 - isol. 98 (IST7), lane 24 - isol. 99 (IST1), lane 25 - isol. 100 (IST8)(very faint band), lane 26 - isol. 101 (IST1), lane 27 - *S. typhimurium* ACM 3598, lane 28 - *S. sofia* Bt6

Electrophoresis of 5µl of PCR product from the DIG-labelling reaction followed by detection of the product proved that a labelled probe was being synthesized. Therefore, only the binding of the probe to target sequence remained to be tested. Hydridizations were performed at the stringent temperature of 68°C and also at the less stringent 42°C. If sequence differences of the target area for the probe existed between *S. virchow* and *S. typhimurium* the use of less stringent conditions should allow the probe to anneal. However, hybridization at 42°C did not result in any detection of probe binding by the *S. virchow* isolates or the *S. typhimurium*

digested genomic DNA, which was the same DNA that the probe was synthesized from. Despite the systematic approach to correct the IS*200* probing difficulty, it was not achieved and only 63 isolates were IS*200* typed.

S. virchow strains have been IS*200* typed previously and shown to possess 0-2 copies of the IS*200* (Gibert *et al.*, 1990). To eliminate the possibility that the *S. virchow* of this study did not contain IS*200* sequences a touch-down PCR was done on 14 isolates using the IS*200*f and IS*200*r primers. The fourteen isolates were from varied sources and included a combination of seven *S. virchow* isolates which had shown hybridizing bands and therefore been assigned an IS*200* type. The remaining seven isolates had not demonstrated hybridizing bands and had not been assigned an IS*200* type. The seven IS*200* typed isolates were 22, 76, 87, 91, 93, 99 and 100. Isolates 29, 33, 34, 44, 50, 54 and 56 were those non-IS*200* typed isolates chosen for PCR screening. The disadvantage of using PCR to detect IS*200* is that only the presence or absence of the insertion element can be determined, it does not provide any indication of the number of copies in the genome. The template concentration (1 µg of the target DNA) and magnesium chloride concentration (2mM) optimized for *S. typhimurium* DNA were employed.

For the 14 isolates tested, no amplicons were observed. This suggested that some of the *S. virchow* isolates in this study may not have the IS*200* sequence.

It was hoped a PCR product from one of the *S. virchow* isolates could be obtained and sequenced so the homology between the probe sequence (from *S. typhimurium*) and the target sequence (in *S. virchow*) could be investigated. This was not achieved. However in order to obtain an indication of the likelihood of sequence variation in the IS*200* sequence, an amplicon from *S. typhimurium* ACM 3598 was cloned and sequenced. The sequence of *S. typhimurium* ACM 3598 was then compared to the published IS*200* sequences of the *S. typhimurium* LT2 and SARA 17 strains. Figure 2.7 shows the sequence alignment of the IS*200* sequences of *S. typhimurium* of LT2 and SARA 17 (obtained from GenBank/EMBL) and *S. typhimurium* ACM 3598.

```
ACM 3598        AGTCTATGGAAACCCCCAGCTAGGCTGGGGGTTCCGGAAAGCTTTCAGCTTTAAGCCAGT
SARA17          ----------------------------------------TCAGCTTTAAGCCAGT
LT2             -GTCTATGGAAACCCCCAGCTAGGCTGGGGGTTCCGGAAAGCTTTCAGCTTTAAGCCAGT
                                                          ***************

ACM 3598        TATTAAAACCCCTTTTGATTTGTTAAAACATCTTGCGGTCTGGCAACTGCAAAAGTTCAA
SARA17          TATTAAAACCCCTTTTGATTTGTTAAAACATCTTGCGGTCTGGCAACTGCAAAAGTTCAA
LT2             TATTAAAACCCCTTTTGATTTGTTAAAACATCTTGCGGTCTGGCAACTGCAAAAGTTCAA
                ************************************************************

ACM 3598        CAAGAAATCAAAAGGGGGTCCCAATGGGGGACGAAAAGAGCTTAGCGCACACCCGATGGA
SARA17          CAAGAAATCAAAAGGGGGTCCCAATGGGGGACGAAAAGAGCTTAGCGCACACCCGATGGA
LT2             CAAGAAATCAAAAGGGGGTCC-AATGGGGGACGAAAAGAGCTTAGCGCACACCCGATGGA
                ******************** ****************************************

ACM 3598        ACTGTAAATATCACATAGTTTTCGCGCCCAAATACCGAAGACAAGCGTTCTATGGAGAGA
SARA17          ACTGTAAATATCACATAGTTTTCGCGCCCAAATACCGAAGACAAGCGTTCTATGGAGAGA
LT2             ACTGTAAATATCACATAGTTTTCGCGCCCAAATACCGAAGACAAGCGTTCTATGGAGAGA
                ************************************************************

ACM 3598        AGCGTAGGGCAGTAGGCAGCATATTAAGAAAATTGTGTGAATGGAAAAACGTACGAATTC
SARA17          AGCGTAGGGCAGTAGGCAGCATATTAAGAAAATTGTGTGAATGGAAAAACGTACGAATTC
LT2             AGCGTAGGGCAGTAGGCAGCATATTAAGAAAATTGTGTGAATGGAAAAACGTACGAATTC
                ************************************************************

ACM 3598        -TGGAAGCAGAATGTTGTGCAGATCATATTCACATGCTTCTGGAGATCCCGCCGAAGATG
SARA17          -TGGAAGCAGAATGTTGTGCAGATCATATTCACATGCTTCTGGAGATCCCGCCGAAGATG
LT2             CTGGAAGCAGAATGTTGTGCAGATCATATTCACATGCTTCTGGAGATCCCGCCGAAGATG
                 ***********************************************************

ACM 3598        AGTGTGTCGAGTTTCATGGGATATCTGAAGGGTAAAAGTAGTCTGATGCTTTACGAGCAG
SARA17          AGTGTGTCGAGTTTCATGGGATATCTGAAGGGTAAAAGTAGTCTGATGCTTTACGAGCAG
LT2             AGTGTGTCGAGTTTCATGGGATATCTGAAGGGTAAAAGTAGTCTGATGCTTTACGAGCAG
                ************************************************************

ACM 3598        TTTGGGGATCTAAAATTCAAATACAGGAACAGGGAGTTCTGGTGCAGAGGGTACTATGTC
SARA17          TTTGGGGATCTAAAATTCAAATACAGGAACAGGGAGTTCTGGTGCAGAGGGTACTATGTC
LT2             TTTGGGGATCTAAAATTCAAATACAGGAACAGGGAGTTCTGGTGCAGAGGGTACTATGTC
                ************************************************************

ACM 3598        GATACGGTGGGTAAGAACACGGCGAAGATACAGGACTACATAAAGCACCAGCTTGAAGAG
SARA17          GATACGGTGGGTAAGAACACGGCGAAGATACAGGACTACATAAAGCACCAGCTTGAAGAG
LT2             GATACGGTGGGTAAGAACACGGCGAAGATACAGGACTACATAAAGCACCAGCTTGAAGAG
                ************************************************************

ACM 3598        GATAAAATGGGTGAGCAATTATCGATCCCGTATCCGGGCAGCCCG-TTTACGGGCCGTAA
SARA17          GATAAAATGGGTGAGCAATTATCGATCCCGTATCCGGGCAGCCCG-TTTACGGGCCGTAA
LT2             GATAAAATGGGTGAGCAATTATCCATCCCCTATCCGGGCAGCCCGATTTACGGGCCGTAA
                ********************** ***** *************** **************

ACM 3598        GTAACGAAGTTTGATGCAAATGTCAGATCGTATGCGCCTGTTAGGGCGCGGCTGGTAAGA
SARA17          GTAACGAAGTTTGATGCAAATGTCAGATCGTATGCGCCTGTTAGGGC-------------
LT2             GTAACGAAGTTTGATGCAAATGTCAGATCGTATGCGCCTGTTAGGGCGCGGCTGGTAAGA
                ********************************************

ACM 3598        GAGCCTTATAGGCGCATCTGAAAAACCTCGGCTAT---------------
SARA17          ------------------------------------------------
LT2             GAGCCTTATAGGCGCATCTGAAAAACCTCGGCTATGCCGGAGGATATTTA
```

Figure 7: CLUSTAL W(1.5) multiple sequence alignment of the *S.typhimurium* sequence data obtained for ACM 3598 to sequence data from Genbank and EMBL for the other *S.typhimurium* strains.

2.3. f) Application of typing methods to outbreak isolates

Nine isolates of *S. virchow* were provided by State Health, Queensland and used to test the efficiency of plasmid profiling and ribotyping as typing tools. The isolates were obtained from faecal specimens of nine individuals involved in a case of food poisoning from a single restaurant.

All nine of the isolates underwent examination for low molecular weight plasmids (refer to Section 2.2.d i) and none possessed any detectable plasmid DNA.

Ribotyping (refer to Section 2.2f) showed that all of the outbreak isolates were of the same ribotype. In addition, their ribotype pattern was different to the patterns of all other *S. virchow* tested in this study. The pattern of the food-borne isolates was assigned ribotype 8. Figure 2.8 shows the ribotyping blot of all of the isolates from the food poisoning incident.

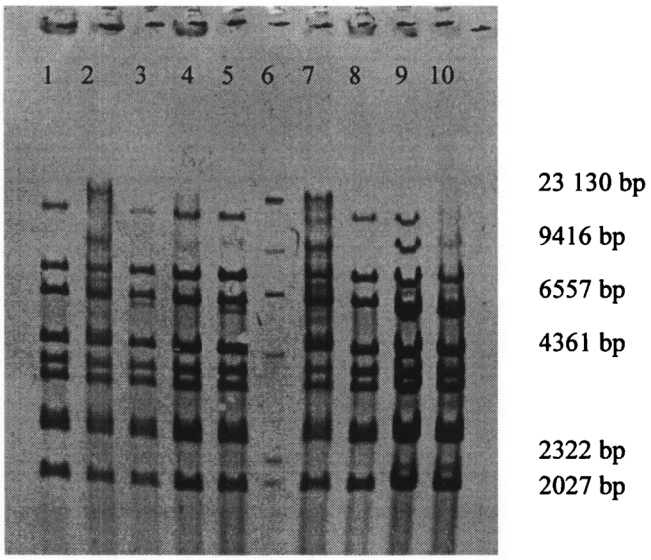


Figure 2.8 - Ribotyping blot of the food poisoning outbreak isolates. Lane 1 - isol. 121 (RT8), lanes 2 - isol. 122 (RT8), lane 3 -isol. 123 (RT8), lane 4 - isol. 124 (RT 8), lane 5 - isol. 125 (RT8), lane 6 - DIG labelled *Hind*III digested Lambda DNA, lane 7 - isol. 126 (RT8), lane 8 - isol. 127 (RT8), lane 9 - isol. 128 (RT8), lane 10 - isol. 129 (RT8)

2.4 Discussion

This study of *S. virchow* was the first to apply a combination of phenotypic and genotypic typing methods to Australian isolates. Every year during the period 1988 - 1995, *S. virchow* was in the top six *Salmonella* serovars isolated from humans in Australia, (National Salmonella Surveillance Scheme, 1988, 1989, 1990, 1991, 1992, 1993a, 1994a). In addition, every year during that period greater than 77% of the *S. virchow* isolations were from Queensland. To accurately reflect the distribution of *S. virchow* in Australia, 75% of the isolates for this study were from various sources in Queensland including humans and poultry.

Five typing methods including phenotypic and genotypic methods were applied to the group of 95 isolates. The five methods were antibiotic susceptibility testing, phage typing, plasmid profiling, ribotyping and IS*200* typing. Ribotyping had not been reported in the literature for the typing of *S. virchow* isolates previously.

There were three research objectives of the epidemiological investigation of ninety-five *S. virchow* isolates. The first was to determine the level of discrimination that each of the methods could achieve in typing *S. virchow*. To quantitate the discrimination for each of the methods the Simpson's index of diversity was applied and a single numerical index of discrimination (D) for each method was calculated (Hunter & Gaston, 1988) (see appendix 3 for an example of the calculation). This index of discrimination is based on the statistical probability that two unrelated isolates would be placed into separate groups by a typing method. For example, a D value of 0.8 indicates that if two unrelated isolates were randomly sampled they would be placed in two separate groups on 80% of occasions. Therefore the range of D values is from 0-1, with 0 being the least and 1 being the most discriminatory.

The second objective was to identify clonal connections between isolates of the *S. virchow* group. Clones are defined as bacterial cultures which are very highly related and therefore are identical when tested by various phenotypic and genotypic typing methods (Orskov & Orskov, 1983).

The results of all five methods in this study were compiled to facilitate the identification of highly related isolates. The isolates which were indistinguishable by all of the methods represented clonal lines of *S. virchow*.

The third objective was to determine if any of the clonal lines of *S. virchow* confirmed the hypothesis that poultry is a source of human infection by *Salmonella* and in particular *S. virchow*. Publications from the United Kingdom and Netherlands (Barrell, 1987; Reilly *et al.*, 1988; Giessen *et al.*, 1991) have reported high and increasing incidence of *Salmonella* in poultry flocks and have indicated that this is the cause of increases in the number of human cases. These conclusions were made on the basis of serotyping and phage typing, which demonstrated that the same serotypes were present in both poultry and humans. Further, Olsen *et al.* (1992a, b) demonstrated that *S. berta* was endemic in the Danish poultry population in the mid to late 1980s and at the same time was the third most common cause of human infection. Comparison of plasmid profiles of the *S. berta* poultry and human isolates was used to confirm that poultry were the cause. Unlike these studies the distinction of clonal lines in the current study was defined as only isolates which were indistinguishable by all five methods tested.

2.4. a) Antibiotic Susceptibility Testing

The seven antimicrobial agents that were tested were chosen because of their spectrum of activity. Turnidge and Stockman (1991) produced a list of the antimicrobial agents recommended for the various testing methods and for different groups of bacteria. Four of the antibiotics recommended for Gram negative bacteria were chosen: ampicillin, trimethoprim/ sulphamethoxazole, gentamicin and ciprofloxacin. Chloramphenicol was also included in the testing regime because it has activity against a wide range of bacteria including *Salmonella*. Furazolidone was the seventh antibiotic included because it is a member of the group of antibiotics termed nitrofurans and these antimicrobial agents are used in the poultry industry outside of Australia.

Thirty of the seventy-four isolates tested (40.5%) demonstrated resistance (including intermediate resistance) to one or more of the antimicrobial agents including furazolidone. Of the thirty isolates demonstrating resistance to one or more agent, 17 (56.7%) were human clinical isolates and nine were animal isolates. Two of the animal isolates were the most resistant isolates in the group. Isolate 68 was resistant to six of the seven agents, furazolidone being the only antimicrobial agent to which it was susceptible. This isolate was from porcine pus, collected from a feral pig that died on a property in the Oonoonba area. The pig was recorded as being ill at the time of death but no treatment with antibiotics was recorded. The explanation for resistance profile of this isolate is unknown. Isolate 62, from a wallaby lymph node, had resistance to five of the agents.

Isolate 55 was a human faecal isolate resistant to four agents, ten isolates were resistant to three agents, eight isolates were resistant to two and nine were resistant to one of the antimicrobial agents tested.

Tetracycline was the antimicrobial agent for which resistance was most common in the present study. Ninety three percent of the strains with intermediate resistance or above demonstrated resistance to tetracycline. This was difficult to explain because tetracycline is not often used in the treatment of *Salmonella* infections. However, tetracycline resistance had previously been reported in *Salmonella* including *S. virchow* (Hadfield *et al.*, 1985; Ward *et al.*, 1990; Threlfall *et al.*, 1993; Mirza *et al.*, 1996; Ramos *et al.*, 1996). Ward *et al.* (1990) reported the incidence of multi-drug resistance in salmonellae, including *S. virchow* in England and Wales. The most common resistance pattern shown by *S. virchow* in both 1981 and 1988 included tetracycline in addition to chloramphenicol, streptomycin, kanamycin, trimethoprim, and furazolidone.

The second most common resistance in the present study was ampicillin, with thirteen isolates resistant. Gentamicin resistance was present in twelve isolates and eight were resistant to chloramphenicol. Three isolates each showed resistance to furazolidone, ciprofloxacin or the combination of trimethoprim/sulphamethoxazole.

Antibiotics from the group called furans, such as furazolidone and nitrofurantoin, have been employed as feed additives in the poultry industry overseas. Their use has been illegal in the Australian industry since January 1994 (Cox *et al.*,

1996a). It was of interest to test resistance to one of these agents with this group of *S. virchow,* since it consisted of a large proportion of poultry isolates. Thirty-one poultry isolates were tested and none were resistant to furazolidone.

The very low incidence of strains resistant to antibiotics from the furan group, both in the poultry and human populations, is perhaps an indication that the ban on the use of furans in the local poultry industry has meant that *S. virchow* in Australia has not been subjected to the selective pressure that strains elsewhere have. In contrast, Ward *et al.* (1990) reported that 50% of *S. virchow* isolates from England and Wales were resistant to furazolidone and that this was possibly due to the use of this drug in chickens. Threlfall *et al.* (1993) also reported an alarming incidence of furazolidone resistance in the United Kingdom. That study indicated that in 1990, 74% of human and 24% of poultry *S. virchow* isolates were resistant to furazolidone and hypothesized a direct link between the use of nitrofurans in the poultry industry.

Antimicrobial susceptibility testing is limited in its value as an epidemiological tool because the resistance factors are often carried on plasmids and can be easily lost or acquired (Threlfall & Frost, 1990; Olsen *et al.*, 1993). Therefore, if epidemiologically results are sought testing should be performed immediately after isolation. This was not the case in the present study and in particular the furazolidone testing was performed much later than testing of the other six antimicrobials.

There was no correlation between antibiotic resistance and the possession of low molecular weight plasmid DNA for the isolates studied. This suggested that the resistance traits observed in these isolates were encoded either on the genome or on large plasmids. Preliminary investigation of the *S. virchow* isolates for high molecular weight plasmids was performed in this study but the results were inconclusive (data not shown).

Antimicrobial susceptibility testing resulted in the isolates being divided into seventeen groups based on the resistances each isolate expressed. An index of discrimination of 0.640 was calculated for susceptibility testing, indicating that two unrelated isolates would be placed in separate groups on 64% of occasions.. This was the highest discrimination index of all five typing methods tested. This level of discrimination was achieved because of the spread of isolates across the groups. Resistance type zero contained all of the isolates which were susceptible to all of the antimicrobial agents tested. Resistance type 1 was isolates with resistance to Furazolidone alone, resistance type 2 was isolates resistant to Gentamicin and Tetracycline only and so forth so that each of the different resistance profiles observed was included. Figure 2.9 is a graphic representation of the data contained in Table 2.3.

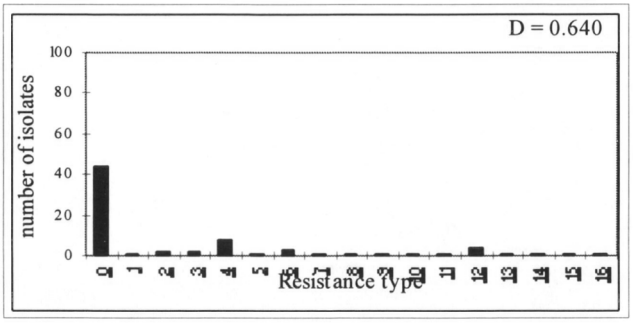


Figure 2.9 - Graph of the distribution of *S. virchow* isolates by resistance types

2.4. b) Phage typing

Chambers *et al.* (1987) established a scheme of 13 standard phages for the typing of *S. virchow.* This set of phages was not available in Australia at the time of this study and therefore, seventy-nine of the *S. virchow* isolates were phage typed in collaboration with the *Salmonella* Reference Laboratory - Public Health Laboratory Service - Colindale, London.

The United Kingdom (UK) study in which the *S. virchow* phage typing set was first described (Chambers *et al.*, 1987), showed that the four most prevalent

phage types were PT8 (25.8%), PT26 (25.2%), PT31 (12%) and PT2 (11.7%). In the current study of Australian *S. virchow*, thirteen phage types were recognized including three subtypes (8a, 34a and 7a). Phage type 8 was the most prevalent phage type in this study at 68.3%. However unlike the UK study, phage types 26 and 2 were not present in the *S. virchow* isolates from Australia and only three isolates (3.8%) of phage type 31 were identified. Therefore it would seem that the distribution of phage types in these two geographically separated groups is quite distinct.

A group of related isolates was highlighted by the phage typing. The identification of isolates 1, 2 and 34 as the only isolates of phage type 31 correlates with their source information. Isolates 1 and 34 were the only isolates in the group of *S. virchow* from feed ingredients used in the poultry industry (meat/bone meal and soya bean meal, respectively) and were typed together by phage typing. The third member of the group, isolate 2, was from imported frog's legs.

The predominance of one phage type emphasized a limitation of using only one typing method. Once a particular phage type becomes dominant it is very difficult to obtain insight into relatedness using that method. This was noted by Olsen *et al.* (1993) who cited the example of the increase in incidence of *S. enteritidis* PT4 in many countries around the world. As a result of this phage type being predominant worldwide, any detection of it can no longer provide evidence of its likely origin. Likewise, the results from this group of Australian *S. virchow* isolates showed that phage type 8 was the dominant phage type and because this is a predominant type elsewhere in the world there is a limitation to the conclusions that can be made based on this method alone.

Another limitation of phage typing is the potential for the phage type of an isolate to change. The changing of phage type has been demonstrated in *S. enteritidis* in which the acquisition of plasmid DNA or loss of the O antigen effects the phage type. When *S. enteritidis* phage type 4 strains acquire plasmid DNA they can become phage type 24. Also loss of O antigen results in a change from phage type 4 to phage type 7 (Holt *et al.*, 1984).

The discriminatory index for phage typing was calculated to be 0.530. This was the second most discriminatory method after antimicrobial susceptibility testing.

Phage typing resulted in 14 groups when each of the subgroups was considered separate (phage type 8 and 8a were considered as two groups). Figure 2.10 is a graph showing the spread of isolates in these groups.

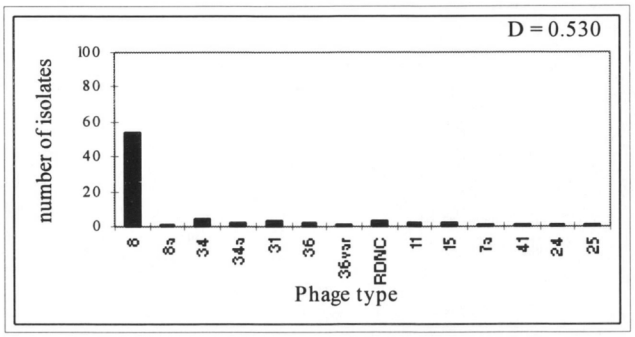


Figure 2.10 - Graph of the distribution of *S. virchow* isolates by phage types

2.4. c) Plasmid Profiling

The most suitable application of plasmid profiling as a typing method is in a confirmatory role. Plasmid profiling can be used to add supporting evidence by being applied to strains which have already been linked by other methods. If strains which are suspected of being related can be shown to have a distinct plasmid pattern and all implicated strains share the same pattern, this is strong evidence that the strains are related. However the ability of plasmid profiling to indicate relationships between strains can be affected by the mobility of plasmid DNA. Plasmids can be transferred between strains and therefore plasmid profiling should be considered in combination with other typing methods.

A technical consideration of plasmid profiling is the existence of plasmids in three forms. As encountered in this study, and referred to in the literature (Olsen *et al.*, 1993), plasmid DNA is present in three forms, closed circular, open circular and nicked. Each of these forms has a different three dimensional structure which results in different relative mobility in an agarose gel and each form appears as a distinct band. In isolates where a number of plasmids are present the resulting banding

pattern will be made more complex by the presence of more than one form of each of the plasmids.

When comparing plasmid profiles different plasmids may appear to be the same if they are similar in size. Under these circumstances, plasmid fingerprinting can be applied to ascertain whether the plasmids are the same or different. Fingerprinting is the use of restriction enzyme analysis of the plasmid DNA to obtain a pattern of the resulting fragments. Plasmids that are the same result in the same pattern. However plasmids that are different at the nucleotide sequence level will demonstrate different restriction patterns. Fingerprinting was used in the present study to confirm the members of plasmid profiles 1 and 2.

Twenty-three percent of the 95 *S. virchow* isolates tested in this study possessed one low molecular weight plasmid. None of the isolates were shown to have more than one low molecular weight plasmid. The actual size of the plasmids were not determined because the aim was to use the presence, absence and number of plasmids to differentiate profiles. There were five plasmid profiles (PP) identified and assigned PP1 - PP5. Plasmid profile 6 was assigned to those isolates tested which did not harbour plasmids.

Sixty-eight percent of the isolates with plasmids were assigned to PP1. Digestion of plasmid DNA from this group resulted in identical restriction patterns indicating that the same plasmid DNA was present in all members of the group. The members of PP1 were exclusively chicken meat isolates from Queensland isolated during the period from September 1991 to February 1993. This is an example of plasmid profiling supporting epidemiological information to confirm a group of related isolates. The details of the original source of each of these chicken meat isolates was not available because of confidentiality agreements, however it is proposed the these isolates were from a common source. It is conceivable that either the farm environment from which these chickens were obtained, or the chickens themselves, were a reservoir of *S. virchow* which carried and maintained this plasmid.

The isolates that formed PP2 also had a common source. The three members of PP2 (43, 44 and 45) were all isolated from sewage effluent collected at Penrith, Sydney, in April 1992. Again plasmid fingerprinting showed that the plasmid DNA

of all three isolates were identical. The epidemiological relevance and specificity of this group was emphasized by the exclusion of isolate 46. Isolate 46, also from sewage effluent, was isolated two months later from a different location in Sydney. This isolate was typed as a member of PP6 because there was no detectable plasmid. Assuming there had been no loss of plasmid from isolate 46, the differentiation between the Penrith isolates and isolate 46 by plasmid profiling was highly discriminatory.

Preliminary work was performed to determine if any of the *S. virchow* isolates harboured large plasmids (data not shown). No conclusive evidence of high molecular weight plasmid DNA was observed.

The index of discrimination for plasmid profiling was 0.360. This calculation was on a total number of 104 isolates because of the inclusion of the nine outbreak isolates. The results of plasmid profiling showed, as other methods had, the predominance of one particular profile, in this case plasmid profile 6. Figure 2.11 shows the plasmid profiles and the distribution of isolates graphically.

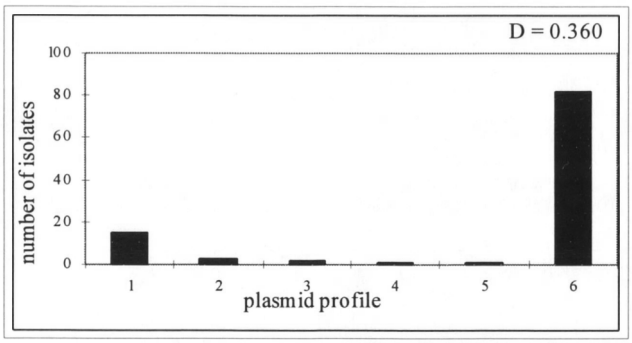


Figure 2.11 - Graph of the distribution of *S. virchow* isolates by plasmid profiles

2.4. d) Ribotyping

Ribotyping is the analysis of bacterial chromosomal DNA using a nucleic acid probe which targets the rRNA genes. The nucleotide sequence of rRNA genes of bacteria are very stable and well conserved and all bacteria have at least one copy.

The discrimination between strains is by comparison of the pattern of DNA fragments, resulting from genomic digest, which bind to the labelled 16S rRNA probe. The number of bands in the pattern is the result of the number of copies of the ribosomal genes on the genome and the nucleotide sequence variation between isolates in the regions of the ribosomal genes.

Many restriction enzymes had previously been employed to digest the genomic DNA for ribotyping of other *Salmonella* serovars. Therefore an initial trial of several enzymes namely, *Pst*I, *Hin*dIII, *Sma*I and *Ban*I was performed. *Ban*I resulted in the most readable patterns for *S. virchow* DNA. In this study a readable pattern was defined as one with few enough bands that the pattern could be distinguished by eye but enough bands that differences between isolates were observed. *Ban*I had not been extensively used for ribotyping previously, but it has been successfully applied to the ribotyping of *Salmonella infantis* (Pelkonen *et al.*, 1994). However there is at least one *Ban*I restriction site present in the 16S rRNA gene of some *Salmonella* serovars as determined by analysis of 16S rRNA sequences lodged with GenBank (NCBI) for *S. paratyphi*, *S. sofia* and *S. typhimurium*. *S. typhimurium* has a *Ban*I site at the nucleotide positions of 1024-1029. There has been no sequence of the 16S rRNA gene for *S. virchow* published but it is possible that a *Ban*I restriction site is present. A restriction site in the rRNA gene in addition to other sites in the region of the rRNA genes and the number of copies of the rRNA genes results in the number of bands which hybridize the 16S rRNA probe. Therefore readable patterns may result but conclusions regarding the number of copies of the rRNA genes can not be directly made.

Eight ribotypes were assigned to the 103 *S. virchow* typed, including the nine isolates from the food-borne outbreak. Ribotype 1 contained 87% (82/94) of the isolates. This included 86.4% (38/44) of all the human isolates and 97% (29/30) of the poultry isolates. This result addressed the third objective of this investigation. The grouping together of almost all of the poultry and human isolates into one ribotype indicated a high level of relatedness between these isolates. This relatedness supported the hypothesis that poultry is a reservoir for human infection.

This hypothesis is further supported by the finding that all of the isolates from infected people with recent travel histories or those who were known to have acquired their infection overseas, were allocated to ribotypes other than RT 1. Ribotype 1 contained exclusively local isolates. In contrast the two members of ribotype 3 were both isolates from individuals associated with travel, with isolate 4 acquired overseas and isolate 84 from a person who had travelled to Fiji. Isolate 87 was the only member of ribotype 6 and was from a person who had travelled to Africa. These ribotyping results suggested that ribotypes 3 and 6 were not native to Australia but instead had been introduced.

The nine outbreak isolates also formed an exclusive and unique ribotype, RT 8. This was conclusive evidence that these isolates were highly related to each other and distinct from other *S. virchow*. Demonstration of the same ribotype for isolates from the suspected food would have confirmed the source of the outbreak.

Ribotype 2 was also a small distinct group which contained the only two isolates from poultry feed components and an isolate from imported frog's legs. This ribotype was a particularly significant finding because it supported the grouping of isolates 1, 2 and 34 by phage typing. These three isolates were the only phage type 31 isolates and the only ribotype 2 isolates. Also the source data indicated that a relationship was likely, at least between isolates 1 and 34.

Ribotype 4 was the only ribotype, other than ribotype 1, which contained local human isolates of *S. virchow*. This ribotype contained isolates 56 and 100 which were from human faeces and isolate 58 from seawater in Bali. This grouping also highlighted the ability of ribotyping to disprove suspected relatedness between isolates. The same individual was the source of isolate 100, RT4 and isolate 83, RT1. These two isolations were five months apart and the assumption was that a persistent infection had been present. However the ribotyping indicated that the isolates were different and therefore it was likely that the individual had not had a persistent infection with the same strain but had suffered re-infection with a second strain of *S. virchow*, during the intervening five month period.

Ribotypes 5 and 7 were represented by only one isolate each and they were from chicken and sheep faeces, respectively. Isolate 33 (RT3) was one of only three chicken isolates which were obtained from the Egg Industry and Research

Development Council (EIRDC) farm survey project and isolate 98 (RT7) was the only sheep isolate in the group of *S. virchow*. The low occurrence of these ribotypes may be due to a low incidence or it may be an artefact of the groups composition.

A ribotyping result was not obtained for isolate 12. Several extractions of the genomic DNA were performed but none resulted in DNA which could be digested by *Ban*I. A lane of unsuccessfully digested was included on blots and probed, but hybridization with the probe never occurred. The reason for this was not established and no similar difficulty has been reported in the literature.

The discriminatory index calculated for ribotyping was 0.359. This calculation included the nine outbreak strains as was done for the plasmid profiling. This index was lower than expected. However despite the low index, ribotyping differentiated the isolates into groups which showed strong correlation with the source data. The ribotyping results also supported the results of phage typing for at least one group and confirmed that human and poultry isolates are highly related.

Figure 2.12 shows the number of ribotypes identified and the spread of isolates across those groups.

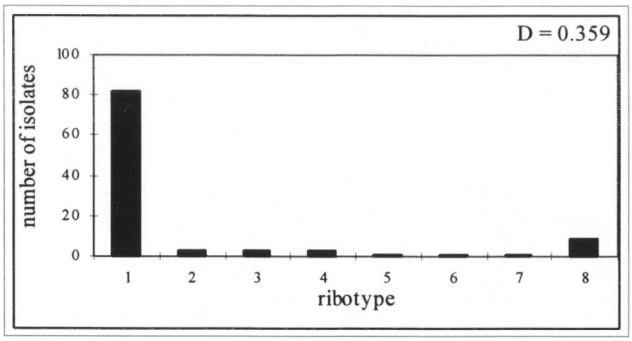


Figure 2.12 - Graph of the distribution of *S. virchow* isolates by ribotyping

2.4. e) *IS*200 typing

IS200 typing utilizes the almost *Salmonella*-specific insertion sequence IS*200* (IS*200* does occur in some *E. coli* and *Shigella* spp.) to discriminate between strains based on the copy number of the insertion sequence and restriction profile of the genomic DNA containing these sequences. The restriction of genomic DNA in this study was done with *Ban*I, after attempts with *Pst*I, *Bgl*II, and *Pvu*II on a subgroup of isolates demonstrated a lack of discriminatory power. This lack of discrimination was defined by the inability to obtain any distinguishing profiles. *Ban*I had not been previously used for IS*200* typing of any *Salmonella* strains in the literature (Gibert *et al.*, 1990; Torre *et al.*, 1993).

*Ban*I, like the other enzymes listed above, could be used for IS*200* typing because its recognition sequence is not present in the IS*200* sequence (Stanley *et al.*, 1991, 1992a; Pelkonen *et al.*, 1994). Therefore the number of bands in the resulting IS*200* pattern is an estimation of the number of copies of the insertion sequence on the chromosome. However, it is not definitive because it is possible that some of the larger DNA fragments that result from genomic digestion could contain more than one copy. A strain may also harbour a large plasmid which has a copy of IS*200* and such large plasmids can be co-extracted with genomic DNA. However the occurrence of IS*200* on plasmids has been reported to be rare (Stanley *et al.*, 1991, 1992b). Torre *et al.* (1993) tested 57 type strains of *S. virchow* phage types and did not detect an IS*200* loci on plasmid DNA in any of the strains.

The application of this method to *S. virchow* isolates as a typing tool was only partially successful because it was not able to be applied to the entire group. There were 63 isolates for which results were obtained before difficulties prevented the remainder of the isolates being IS*200* typed (see section 2.3 e).

Eighty-nine percent of the 63 isolates typed were IS*200* type (IST) 1. Only seven isolates showed alternative banding patterns. Isolates 1, 2, 11, 62, 87, 98 and 100 all showed alternative patterns, each of which was unique forming ISTs 2-8.

The discrimination index for IS*200* typing was the lowest of the five methods at 0.211. This was expected because less isolates were typed by this method and

almost 90% of those typed belonged to IST2. Figure 2.13 shows graphically the predominance of IST1.

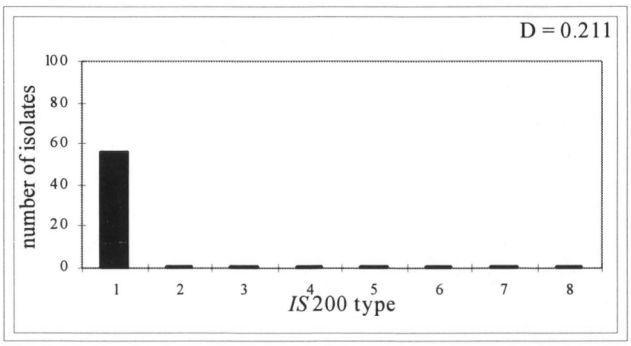


Figure 2.13 - Graph of the distribution of *S. virchow* isolates by IS*200* typing

The results of IS*200* typing indicated that the copy number of IS*200* in *S. virchow* is low because the patterns consisted of only one to four bands. Torre *et al.* (1993) investigated 57 type strains of *S. virchow* phage types and found only six strains carried IS*200* sequences. These six strains formed three IS*200* types and the number of bands in these types were one, four and five. The study did not directly associate the number of bands with copies of IS*200* but instead defined the profiles by the molecular weights of the bands which were detected. Gibert *et al.* (1990) tested two *S. virchow* strains, one of which had 1 copy and the other had 2 copies of IS*200*. IS*200* typing of *Salmonella enteritidis* and *Salmonella dublin* has also found low copy numbers and report that it limits the usefulness of the technique as a typing tool (Baquar *et al.*, 1993).

Nevertheless, IS*200* typing has proved informative in some studies despite the low copy number of the element. In particular, Stanley *et al.* (1991, 1992b) have described recent evolutionary changes within *S. enteritidis* and identified three clonal lineages, using IS*200* typing.

Reports that some *S. virchow* strains (Torre *et al.*, 1993) and other serovars of *Salmonella* (Gibert *et al.*, 1990) do not have IS*200* sequences, lead to the inclusion of PCR detection in the current study.

Fourteen *S. virchow* isolates were tested by touchdown PCR and no amplicons were detected, suggesting that no IS*200* sequence was present. Several of

these isolates (22, 76, 87, 91, 93, 99 and 100) had previously shown at least one hybridizing band in the southern blots indicating that there were IS*200* copies present. The conditions for the PCR reaction were optimized using genomic DNA from *S. typhimurium* ACM 3598 which is known to carry 6-10 copies of IS*200* (Lam & Roth, 1983; Deonier, 1987; Haack & Roth, 1995). A difference in the copy number of IS*200* between *S. typhimurium* and *S. virchow* could have contributed to the failure of the PCR to detect IS*200* in the *S. virchow* isolates. Using a higher concentration of template DNA may have rectified this problem.

A second potential reason for the failure of amplification is that the IS*200* sequences of *S. typhimurium* and *S. virchow* are different and thus the PCR conditions and primer sequences used with *S. typhimurium* are not optimal for *S. virchow*. There is some evidence to support the differences in nucleotide sequence of IS*200*. Bisercic *et al.* (1993) reported *S. typhimurium* strains with hybridizing copies of the IS*200* but the element could not be detected by PCR (similar to the results in this study for isolates 22, 76, 87, 91, 93 and 99). The hypothesis was that the PCR was failing due to primers not annealing as a consequence of sequence variation at the primer annealing positions. The hypothesis was supported by constructing a second reverse primer which was four bases shorter than the original. Using this primer and the original forward primer, an amplification product of the correct size was obtained (Bisercic & Ochman, 1993).

The primers that were used in the present study of *S. virchow* isolates were those of Baquar *et al.* (1993). These primers were designed from the *S. typhimurium* sequence available at the time. It is therefore possible that differences between the IS*200* sequence of *S. virchow* and *S. typhimurium* resulted in the primers being unable to anneal.

A third suggestion for the unsuccessful PCR amplification relates to the secondary structure of the target DNA. Gibert *et al.* (1991) described three potential hairpin loops in the secondary structure of the IS*200* sequence. The largest of these is centered at position 688 and two others are at positions 608 and 655. The reverse primer used in the present study spans the region 689-708. It is possible that the secondary structure of the IS*200* prohibits the reverse primer from binding efficiently and therefore no amplification occurs.

In an effort to minimize the effect of the secondary structure in IS*200* a touchdown PCR protocol was employed. This involved early cycles of the PCR at high annealing temperatures (60°C) to minimize secondary structure formation at the target site during annealing of the primers. The annealing temperature was then decreased stepwise and thirty cycles were completed at a low annealing temperature (50°C). The use of touchdown PCR in this study did not result in amplification of the IS*200* element from any of the 14 *S. virchow* isolates tested.

The generation of an IS*200* amplicon from *S. virchow* would have allowed cloning and sequencing of the element and lead to the first non - *S. typhimurium* IS*200* sequence data. This data would have provided an indication of the level of homology between the IS*200* of *S. typhimurium* and *S. virchow*, but no PCR product from *S. virchow* was obtained. Nevertheless the PCR product of *S. typhimurium* ACM 3598 was cloned and subcloned using the IS*200* internal *Eco*RI site and sequenced.

Bisercic *et al.* (1993) had reported that the IS*200* sequence of the *S. typhimurium* SARA strain 17 was different at six positions to the original sequence reported by Gibert *et al.* (1991) for *S. typhimurium* LT2. Comparison of the sequence data deposited in GenBank (NCBI) and the European Molecular Biology Laboratory (EMBL) by these two research groups could only identify five differences over the regions analyzed by both. Regardless, the sequence data for *S. typhimurium* ACM 3598 obtained in this study had 100% homology at the nucleotide level with the sequence data of Bisercic *et al.* (1993) for SARA 17. The sequence of ACM 3598 determined in this study contained precisely the same five mismatches when compared with the original sequence of Gibert *et al.* (1991) (see Figure 2.7). This result suggested that the IS*200* sequence is well conserved, at least within a serovar. Therefore any future work should investigate the template and secondary structure restrictions of the current PCR.

2.4. f) Investigation of the food-borne outbreak

The occurrence of a food-borne outbreak of *S. virchow* allowed plasmid profiling and ribotyping to be tested. Plasmid profiling was applied to this group of isolates because they had already been linked by other methods (Queensland State Health had identified them as part of an outbreak). Importantly, these isolates had only recently been isolated. Ribotyping was tested because it had proven very successful in grouping isolates based on their epidemiological information. Phage typing could not be performed on these isolates because they were received too late for shipment to the Laboratory of Enteric Pathogens, Public Health Laboratory Service, London.

All of the isolates obtained from the outbreak were indistinguishable by plasmid profiling and ribotyping. All isolates lacked plasmids but more conclusively they all had the same ribotyping pattern. Significantly, the ribotyping pattern was unique to the isolates of the food-borne outbreak when compared to the seven ribotypes which had already been observed in this study. This indicated that all of the individuals from which these isolates were obtained were infected with the same strain of *S. virchow*. Isolation of strains from the foods which were indistinguishable from the faecal isolates by ribotyping and plasmid profiling would have provided unequivocal evidence of the vehicle of transmission. This investigation highlighted the need for a combined approach to investigations of outbreaks which should include effective and discriminatory methods, comprehensive epidemiological information and co-operation from all persons involved to obtain appropriate samples.

2.4. g) Summary

This section reports for the first time the successful application of five typing methods to a group of Australian *S. virchow* isolates.

The first objective of this section was to determine the discriminatory power of each of the methods. The Simpson's index of diversity was applied to give a single numerical index of discrimination (D) for each method. Table 2.8 shows a summary of the discriminating indices for the methods used as well as the number of isolates tested by each method, number of types each method produced and the percentage of isolates that were placed into the largest type for each method.

Table 2.8 - Summary of the discriminating indices for the typing methods applied to *S. virchow*

Typing method	No. isolates tested	No. of types	Frequency of largest type	Discrimination Index (D)
Antibiotic susceptibility	74	17	59.5%	0.640
Phage typing	79	14*	68.4%	0.530
Plasmid profiling	104	6	78.9%	0.360
Ribotyping	103	8	79.6%	0.359
IS*200* typing	63	7	88.9%	0.211

* 14 phage types when the three subtypes are considered as separate types

Antimicrobial susceptibility testing was the most discriminatory method employed for this particular group of *S. virchow* isolates, followed by phage typing. The reasons for antimicrobial susceptibility testing being so discriminatory was that the different resistance profiles observed meant that a large number of types were assigned. Also this method had the lowest frequency of isolates in the largest type which meant that a larger number of isolates were spread over a large number of types.

Ribotyping, plasmid profiling and IS*200* typing were less discriminatory. The lack of discriminatory power in each of these methods arose because most of the isolates belonged to the dominant type.

The second objective was to identify clonal lines in the group of *S. virchow* and this was achieved by combining the results of all five typing methods. This allowed isolates that were indistinguishable by more than one method to be easily recognized. A clonal line is defined as independent isolates that are highly related as determined by being indistinguishable by several typing methods (Orskov & Orskov, 1983; Christensen *et al.*, 1994). Thirty subgroups of the *S. virchow* isolates were identified when the results were combined. The D value of the combination of methods was calculated to be 0.95. This D value was considerably higher than the D values of any of the methods used independently. Isolates which were indistinguishable by all five methods and identified as highly related at this level of discrimination, represented members of a clonal line. Therefore four clonal lines of *S. virchow* were identified in this study and many other potential members of these clonal lines were present in the group but required confirmation by the completion of one or more method. An example was isolate 54, which presently forms a group on its own, but could belong to the clonal line of isolates including 61, 63, 68, 69, 71, 74 and 77, if its IS*200* type was determined and found to be IST 1. Table 2.9 shows the isolates grouped by clonal lines (shaded) or where some methods were not tested, the isolates are grouped by the available results.

Table 2.9 - Clonal lines of *S. virchow* and other related isolates grouped according typing methods

Isolate	Ribotype	Plasmid Profile	Phage Type	Antibiotic Resistance	IS*200* type	Source
12	NT	6	8	S	NT	Chicken Qld
28	1	1	NT	S	NT	Chicken Qld
75	1	1	NT	S	1	Chicken Liver Qld
8	1	1	NT	S	1	Chicken Qld
27	1	1	8	S	NT	Chicken Qld
29	1	1	8	S	NT	Chicken Qld
30	1	1	8	S	NT	Chicken Qld
15	1	1	8	S	1	Chicken Qld
23	1	1	8	S	1	Chicken Qld
24	1	1	8	S	1	Chicken Qld
25	1	1	8	S	1	Chicken Qld

Isolate	Ribotype	Plasmid Profile	Phage Type	Antibiotic Resistance	IS*200* type	Source
26	1	1	8	S	1	Chicken Qld
76	1	1	8	S	1	Chicken Qld
80	1	1	8	S	1	Chicken Qld
78	1	1	8	R	1	Chicken Qld
41	1	1	23	R	NT	Chicken Qld
44	1	2	NT	S	NT	Sewage NSW
45	1	2	RDNC	S	NT	Sewage NSW
43	1	2	36	S	NT	Sewage NSW
86	1	3	NT	NT	1	Human ACT
42	1	3	8	S	NT	Chicken Qld
52	1	4	8	R	NT	Human Qld
82	1	5	8a	NT	1	Human Qld
21	1	6	NT	S	1	Chicken Qld
48	1	6	NT	R	NT	Chicken Qld
59	1	6	NT	R	NT	Human NSW
66	1	6	NT	R	1	Kangaroo Qld
62	1	6	NT	R	5	Wallaby Qld
57	1	6	NT	NT	NT	Human Qld
67	1	6	RDNC	R	1	Bovine Qld
31	1	6	8	S	NT	Human Qld
39	1	6	8	S	NT	Human Qld
40	1	6	8	S	NT	Human Qld
46	1	6	8	S	NT	Sewage NSW
37	1	6	8	R	NT	Human Qld
36	1	6	8	R	NT	Human Qld
35	1	6	8	R	NT	Human Qld
53	1	6	8	R	NT	Human Qld
3	1	6	8	S	1	Possum
5	1	6	8	S	1	Macadamia Nuts
7	1	6	8	S	1	Human Qld
9	1	6	8	S	1	Chicken Qld
13	1	6	8	S	1	Chicken Qld
14	1	6	8	S	1	Chicken Qld
16	1	6	8	S	1	Chicken Qld
17	1	6	8	S	1	Chicken Qld
18	1	6	8	S	1	Chicken Qld
19	1	6	8	S	1	Chicken Qld
20	1	6	8	S	1	Chicken Qld
22	1	6	8	S	1	Chicken Qld
73	1	6	8	S	1	Human Qld
79	1	6	8	S	1	Human Qld

Isolate	Ribotype	Plasmid Profile	Phage Type	Antibiotic Resistance	IS*200* type	Source
81	1	6	8	S	1	Human Qld
61	1	6	8	R	1	Wallaby Qld
63	1	6	8	R	1	Wallaby Qld
68	1	6	8	R	1	Porcine Qld
69	1	6	8	R	1	Cane Toad Qld/NT
71	1	6	8	R	1	Human Qld
74	1	6	8	R	1	Human Qld
77	1	6	8	R	1	Human NSW
54	1	6	8	R	NT	Human Qld
88	1	6	8	NT	1	Human Qld
90	1	6	8	NT	1	Human Vic
92	1	6	8	NT	1	Human Blood Fiji
96	1	6	8	NT	1	Human Vic
97	1	6	8	NT	1	Human Qld
99	1	6	8	NT	1	Human Qld
11	1	6	8	S	4	Chicken Qld
83	1	6	11	NT	1	Human Vic
85	1	6	11	NT	1	Human Vic
47	1	6	15	S	NT	Chicken Qld
50	1	6	15	R	NT	Human Qld
95	1	6	24	NT	1	Human Qld
101	1	6	25	NT	1	Human Qld
38	1	6	34	R	NT	Human Qld
51	1	6	34	R	NT	Human Qld
60	1	6	34	R	1	Bovine Qld
64	1	6	34	R	1	Kangaroo Qld
72	1	6	34	R	1	Human Qld
89	1	6	34a	NT	1	Human Qld
91	1	6	34a	NT	1	Human Qld
93	1	6	36	NT	1	Human Qld
94	1	6	36(var)	NT	1	Human Qld
34	2	6	31	S	NT	Chicken Feed Qld
1	2	6	31	S	2	Meat/Meal Qld
2	2	6	31	S	3	Frog's Legs Imported
84	3	6	7a	NT	1	Human Tas Travel Fiji
55	3	6	8	R	NT	Human SA

Isolate	Ribotype	Plasmid Profile	Phage Type	Antibiotic Resistance	IS*200* type	Source
4	3	6	8	R	1	Human O/S
56	4	6	NT	R	NT	Human SA
58	4	6	NT	R	NT	Seawater Bali
100	4	6	NT	NT	8	Human Vic
33	5	6	NT	S	NT	Chicken Qld
87	6	6	RDNC	NT	6	Human Vic Travel Africa
98	7	6	NT	NT	7	Sheep Vic

Key

R - resistant

S - sensitive

NT - not tested

RDNC - reacts, does not conform

The largest clonal line contained isolates 3, 5, 7, 9, 13, 14, 16-20, 22, 73, 79 and 81, four of which were human isolates and nine which were chicken isolates. The recognition of this clonal line addressed the third objective of this study. The presence of a clonal line which contained both poultry and human strains confirmed the hypothesis that poultry is a reservoir for human infection. The presence of such highly related isolates in both humans and poultry, which are derived from the same ancestor, proves that these strains have been transmitted from one host to the other. *Salmonella* infection is most commonly by ingestion of contaminated and/or under-prepared foods and therefore it is the poultry which are the vehicle of transmission to humans.

Many other groups of highly related isolates were also apparent when the combined results were tabulated and some of these groups were supported by the source information. Isolates 43, 44 and 45 were all isolated from sewage in Penrith, Sydney in April 1992 and were subjected to four of the five typing methods. All three isolates were indistinguishable by ribotyping (RT 1), plasmid profiling (PP 2) and antibiotic susceptibility (S) and only phage typing was able to differentiate. In

contrast, isolate 46, which was also a sewage isolate but from a different sewage plant (but still in Sydney) and isolated in June 1992, was excluded from the group of sewage isolates based on its plasmid profile (PP 6) and phage type (PT 8).

Another group of highly related isolates were isolates 1, 2 and 34. These three isolates were indistinguishable by four of the methods, with only IS*200* typing able to differentiate them (isolate 34 was not IS*200* typed and so two of these three could be indistinguishable by all five methods). If IS*200* typing were to show that isolate 34 was IST 2 this would make the only two isolates from feed components indistinguishable.

Many of these groups which contain isolates that differ in only one method may also be clonal lines. It has been suggested that if a less rigorous definition of a clone is allowed then isolates which are not completely identical may still be considered a clone (Orskov & Orskov, 1983).

The combined methods also supported the conclusion that isolates 83 and 100 were not the same strain, although they were from the same individual. Both isolates underwent plasmid profiling, ribotyping and IS*200* typing. Ribotyping had shown that the two isolates had different ribotypes and IS*200* typing also put the two isolates into different IS*200* types. The result of IS*200* typing alone was significant in light of the fact that so few isolates were typed by IS*200* and only seven isolates including isolate 100 were not the predominant type. Plasmid profiling was the only method to type isolates 83 and 100 the same but this was PP6 which only indicated that neither isolate harboured any low molecular weight plasmid DNA. Therefore the evidence is strong that this individual had two separate infections with different strains of *S. virchow*.

Additional typing methods not used in this study which may be considered for future research of the epidemiology of *S. virchow* are MEE and PFGE. Schoonmaker *et al.* (1992) and Prevost *et al.* (1992) both demonstrated that PFGE has higher discriminatory power than ribotyping when applied to *Legionella pnuemophila* and *Staphylococcus aureus* respectively. PFGE is more discriminatory than ribotyping because it is based on the heterogeneity of restriction sites throughout the genome, rather than the immediate rRNA gene region.

Questions raised from IS*200* typing suggest that further work should be undertaken on the IS*200* sequence of *S. virchow*. This should include re-designing the PCR primers (initially their position), and sequencing of the IS*200* of *S. virchow* to determine the extent of sequence divergence between serovars. Since PCR and Southern blotting is currently not able to detect the presence of IS*200* in the strains an alternative approach would be to do a dot blot of genomic DNA followed by detection with the DIG-labelled probe. This would allow more genomic DNA to be applied to the membrane and a stronger signal should be obtained.

This study is the first to type a group of Australian *S. virchow* isolates using both phenotypic and genotypic methods. The study employed five typing methods and determined antimicrobial susceptibility testing and phage typing to be the most discriminatory. While ribotyping, plasmid profiling and IS*200* typing were not as discriminatory, ribotyping was found to provide accurate correlation with the available data and highlighted relatedness of many of the isolates. The combination of the five methods was 1.5 times more discriminatory than either antimicrobial susceptibility testing or phage typing alone. This enhanced level of discrimination and the presence of isolates which were indistinguishable by all five methods identified clonal lines of *S. virchow*. One of these clonal lines contained both human and poultry isolates and thereby confirmed that for *S. virchow* in Australia, poultry is a vehicle of transmission to humans.

CHAPTER 3

S. VIRCHOW AND SEF17 FIMBRIAE

Chapter 3 : *S. virchow* and SEF17 fimbriae

3.1 Introduction

Fimbriae are virulence associated surface structures which have been demonstrated to mediate adhesion of bacterial cells to host tissues and also facilitate colonization (Müller *et al.*, 1991).

There are several fimbrial types expressed by *Salmonella* spp. but the functions of most of these in pathogenesis has not been extensively studied. For this study one type of fimbria was investigated, namely SEF17. This particular structure was chosen because it has been demonstrated in *S. enteritidis* and is known to be involved in fibronectin binding by *S. enteritidis* strains (Collinson *et al.*, 1993). Strains expressing these fimbriae are particularly aggregative and suspected of enhanced survival in the gut because of their adhesive fimbriae (Collinson *et al.*, 1993).

Previously, genotypic screening with a probe directed against the *agfA* gene, which encodes the SEF17 structural protein, demonstrated that the gene is widely distributed amongst many serovars of *Salmonella* (Doran *et al.*, 1993). Furthermore, expression of this gene was confirmed by Doran *et al.* (1993) using immunoblotting. Strains of *S. virchow* were not included in the study of Doran *et al.* (1993) and therefore SEF17 fimbriae have not been confirmed in *S. virchow*. The hypothesis for this study was that *S. virchow* possesses SEF17 fimbriae and that they have a role in mediating invasive illness frequently associated with *S. virchow* infection.

3.2 Materials and Methods

3.2. a) Indications of SEF17 production

3.2. a (i) Congo Red binding

Ninety-five *S. virchow* isolates (refer to Table 2.1) were grown on Congo red agar to determine their ability to bind this hydrophobic dye. The agar medium used was T medium (Collinson *et al.*, 1991) containing congo red (Sigma Aldrich Pty Ltd) at a concentration of 100μg/ml (Collinson *et al.*, 1993). Isolates were grown at 37°C for 24 hours, followed by room temperature (ca. 21°C) for a further 6 days (Method 1). *S. enteritidis* 6/E5 was included as a positive control strain because it had been shown previously to bind Congo red (Eglezos, 1994). A positive result was recorded when the colonies took up the red dye from the medium to produce red/orange colonies; a negative result was a pink colony. The positive result was extremely subjective, as the colour ranged from dark red with the surrounding agar cleared of red dye, to colonies with slightly orange centres. This work was repeated by Dr K. Collinson of the University of Victoria, British Columbia on 47 of the isolates (1, 2, 4, 5, 8, 9, 11, 13, 15, 21, 23, 25, 27, 28, 30, 33, 34, 43-46, 52, 54-59, 61-64, 67, 68, 75, 76, 80-84, 86, 87, 92, 97, 98 and 100). Incubation was at both 37°C and room temperature (ca. 21°C) separately and binding was recorded at 1 day, 2 days, 5 days or 11 days (Method 2). *S. enteritidis* 27655 strain 3b was included as the positive control (Feutrier *et al.*, 1986; Collinson *et al.*, 1991, 1993).

3.2. a (ii) Colony morphology on T medium

Ninety-five *S. virchow* isolates (refer to Table 2.1) were grown on T medium (Collinson *et al.*, 1991) as a second phenotypic test to indicate fimbrial expression. Isolates were grown at 37°C for 24 hours followed by room temperature (ca. 21°C) for a further 6 days. Results were recorded as positive when the colonies had a

wrinkled non-mucoid appearance and negative when the colonies were smooth and mucoid. *S. enteritidis* 6/E5 was included as a positive control because it had been shown to express a wrinkled colony morphology (Eglezos, 1994).

3.2. a (iii) Colony morphology on CFA agar

Ninety-five *S. virchow* isolates (refer to Table 2.1) were grown on Colonization Factor Antigen (CFA) agar (Evan *et al.*, 1977). On this media, strains of *S. enteritidis* have been shown to have a contoured colony morphology and this phenotype correlates with the virulence of strains in mice (Eglezos, 1994). Isolates were inoculated onto CFA agar and incubated for 24 hours at 37°C followed by room temperature (ca. 21°C) for 13 days. *S. enteritidis* 6/E5 was included as the positive control because it had been shown to form contoured colonies (Eglezos, 1994). The results were recorded as positive when colonies had a contoured or wrinkled appearance and negative when the colonies were smooth and mucoid.

3.2. a (iv) Growth in static CFA broth culture

Ninety-five *S. virchow* isolates (refer to Table 2.1), excluding isolate 80 were inoculated into 8 ml of colonization factor antigen (CFA) broth (Evan *et al.*, 1977) containing 12mM Na_2HPO_4 and 5mM KH_2PO_4 (Collinson *et al.*, 1991) in glass test tubes. Cultures were incubated without shaking, at 37°C for one week followed by room temperature (ca. 21°C) incubation for a further three days. *S. enteritidis* 6/E5 was included as the positive control. A positive result was the presence of growth as a pellicle on the surface of the broth and a negative result was uniform turbidity throughout the broth.

3.2. b) PCR detection and sequencing of the *agfA* gene

3.2. b (i) Selection of primers

A rapid method to detect the *agfA* gene was developed using PCR methodology. The sequences of the first set of published primers developed for the *agfA* gene were reported by Collinson *et al.* (1993). A second set of primers were published which facilitated subcloning by introducing terminal restriction endonuclease cleavage sites (Doran *et al.*, 1993). Both of these primer pairs included a degenerate forward primer designed to the N-terminus of the *agfA* gene, which incorporated a biased, mixed oligonucleotide sequence. In the present study, to overcome the need for this mixed biased primer, a third primer pair was designed from the *agfA* gene sequence of *S. enteritidis* 27655-3b, available at the time (GenBank Accession Number S65743) (Doran *et al.*, 1993). This was done using the software program - Primer, Version 0.5 (Whitehead Institute for Biomedical Research) available from the Australian National Genome Information Service (ANGIS). The chosen primer pair was designated: Agff, an 18bp oligomer (5' GTCGTACCACAGTGGGGC 3') with a T_m of 60.0°C; and from the opposite strand a 19bp oligomer, Agfr (5' GTTCCACTGGTCGATGGTG 3'), with a T_m of 59.9°C. This PCR primer pair amplified a 258bp product. Before having these primers synthesized a similarity computation was performed using the NCBI BlastN 1.3.13MP network service (Altschul *et al.*, 1990). This was done to search for other known sequences containing regions of similarity to either of the primer sequences. In the case of both primers the only sequence identified with similarity was the *agfA* gene of *S. enteritidis* 27655-3b from which they were designed.

3.2. b (ii) Preparation of PCR template DNA

Boiled cell lysates were used as template DNA for PCR reactions. After overnight culture incubation at 37°C, 1.5ml of tryptone soya broth culture was centrifuged at 17 320*g* for five minutes to pellet the cells and the supernatant

removed. The cell pellet was then resuspended in 1ml of sterile distilled H_2O (sdH_2O) and boiled for five minutes. After boiling, the tubes were again centrifuged at 17 320*g* for five minutes to pellet the unwanted cell debris and the supernatant was transferred to a fresh microcentrifuge tube. The concentration of DNA in the supernatant was determined by the absorbance at 260nm using a spectrophotometer. The concentration of DNA was adjusted to 2μg / 10μl with sdH_2O and 1μg of DNA was added to the PCR reactions. These preparations of DNA template were stored at -20°C.

3.2. b (iii) PCR reaction conditions and detection of product

PCR reaction volumes of 50μl were used, containing the following reagents prepared in a master mix :

template DNA	1μg
10 x buffer containing 15mM $MgCl_2$	5μl
dNTPs (1.25mM)	8μl
Agff primer (40ng/μl)	2.5μl
Agfr primer (40ng/μl)	2.5μl
sdH_2O	27μl
	45μl/reaction

A "hot start" PCR protocol was used in which 1.2U of Tth *plus* DNA polymerase (Biotech International Ltd.) was added to each reaction after an initial denaturation step of 94°C for 4 minutes.

The *agfA* PCR cycling conditions used after the initial denaturation were :

30 cycles of
60°C 1 minute
72°C 2 minutes
94°C 1 minute
↓
1 cycle of
60°C 1 minute
72°C 5 minutes
↓
4°C soak

The resulting PCR products were visualized using agarose gel electrophoresis with 0.7-1.2% agarose gels in 1 x TBE (Sambrook *et al.*, 1989) and UV transillumination after ethidium bromide staining. pBR322 - *BstN1* digested DNA was used as the molecular weight marker.

Isolates 58, 59 and 100 were negative by PCR this protocol and further investigation of these three isolates was performed to ensure optimization of template DNA and $MgCl_2$ concentration had been achieved. PCR was carried out using 0.1, 0.5, 1 and 2μg of template DNA and concentrations of 1.5, 2 and 3mM $MgCl_2$ in a matrix format.

3.2. b (iv) Sequencing of the *agfA* PCR product

Direct sequencing of the PCR products of two *S. virchow* isolates, 5 and 21, was attempted to confirm that the PCR product was from the *agfA* gene and to determine if any sequence variation occurs between *S. enteritidis* and *S. virchow.*

After the PCR reaction was completed the *agfA* PCR product was purified from the unincorporated primers and dNTPs, using the Magic™ PCR Preps DNA Purification System (Promega Corporation). The direct sequencing reactions were performed using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit following the manufacturer's instructions for cycling conditions.

Each isolate was sequenced on both strands. A modification of the phenol/chloroform extraction outlined in the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit protocol was used for the purification of the extension products. The modified extraction protocol was :

1. Reaction mix was removed to a fresh tube and 80µl of sdH_2O added
2. 100µl of phenol/H_2O/chloroform (Applied Biosystems Inc.) was added at room temperature and the tube vortexed briefly, then centrifuged at 17 320*g* for 2 minutes
3. The aqueous phase was transferred to a fresh tube and the phenol/H_2O/chloroform step repeated
4. The aqueous phase was transferred to a fresh tube and the extension product precipitated by adding 10µl of 3M sodium acetate (pH 5) and 300µl of 100% ethanol (-20°C). The contents of the tube were mixed by inversion after each addition and held at -70°C from 30 minutes to overnight
5. The tube was centrifuged at 17 320*g* for 15-30 minutes at 4°C, to pellet the DNA
6. The sodium acetate/ethanol was removed and the pellet washed with 700µl of 70% ethanol (-20°C) and the pellet dried using a Speed Vac concentrator (Savant Instruments Inc.)

Electrophoresis and detection of the sequencing products was performed by the DNA Sequencing Facility, University of Queensland, using the 373A DNA sequencer (Applied Biosystems Inc.). The results were analysed using Simseqed (sequencing editing tool) and ClustalW (1.6) multiple sequence alignment tool available from ANGIS.

3.2. c) Detection of SEF17 fimbriae using electron microscopy

Methodology similar to Collinson *et al.* (1991) was used to visualize individual bacterial cells of *S. virchow* isolate 64 expressing thin, aggregative fimbriae. Several other strains were tested initially including *S. virchow* isolate 57 and *S. enteritidis* phage type 4 strain G7. Fimbrial tufts had previously been shown using negative staining on cells of *S. enteritidis* phage type 4 strain G7 (Eglezos, 1994). However results from Congo red binding indicated that isolate 64 was likely to be the strongest producer of SEF17 and therefore only isolate 64 was investigated throughout.

A colony incubated on T medium for 14 days at 37°C was resuspended in several drops of sdH_2O. Formvar-coated copper grids were held between forceps while a drop of emulsified bacterial cells was added to the grid and left for two minutes to attach. The excess liquid was removed by capillary action with blotting paper and the negative stain added.

Several different negative stains were tried including 1% and 2% ammonium molybdate (pH 6.8) with bacitracin, 1% and 2% uranyl acetate and phosphotungstic acid. The best contrast between bacterial cells, the grid and the fimbriae was obtained using 1% ammonium molybdate containing 0.1% glycerol (spreading agent) at pH 7.2. The pH was adjusted using ammonium hydroxide. The stain was left on the grid for two minutes and the excess drained away by blotting paper. Grids were air dried and observed with a Joel 1010 transmission electron microscope operated at 60kV.

3.2. d) Detection of SEF17 protein by PAGE and immunoblotting

3.2. d (i) Purification of SEF17 protein

A modification of the method of Collinson *et al.* (1991) was employed to purify SEF17 protein from *S. virchow* isolate 64. The method was:

1. Following culture on T medium at 37°C for two days, growth was scraped from one agar plate and suspended in 3ml of Tris buffer containing RNase and DNase, both at a concentration of 1mg/ml
2. Cells were sonicated for one minute and then $MgCl_2$ was added to 1mM concentration and incubated at 37°C for 20 minutes
3. Lysozyme was added to 1mg/ml and the samples were incubated at 37°C for 40 minutes without shaking
4. Sodium dodecyl sulfate (SDS) was added to a concentration of 1% and the samples were incubated at 37°C for a further 30 minutes
5. The remaining insoluble material was collected by centrifugation at 12 100*g* for 15 mins at 25°C
6. The pellet was then resuspended in 3ml of Tris buffer and boiled for 10 minutes to dissolve any agar and the centrifugation was repeated to pellet the insoluble material
7. The pellet was again resuspended in 3ml of Tris buffer and digestion with RNase, DNase and lysozyme was repeated
8. The insoluble material was collected by centrifugation and the pellet washed once in 1ml of Tris buffer and resuspended in 45µl of SDS - PAGE sample buffer (refer to appendix 2)
9. The sample was boiled for 15 minutes, loaded onto a polyacrylamide gel (12% resolving gel and 4% stacking gel (refer to appendix 2)) and subjected to electrophoresis for 5 hours at 20mA

10. The white flocculent material which did not enter the stacking gel was recovered from the wells using a fine syringe and this insoluble cell material containing SEF17 was pelleted by centrifugation at 17 320*g*
11. This pellet was washed with sdH_2O and dehydrated with 95% ethanol, then dried using a Speed Vac concentrator (Savant Instruments Inc.)
12. The dried pellet was resuspended in 100µl of sdH_2O and two volumes of 0.2M glycine. The sample was boiled for 10 minutes and recovered by centrifugation at 17 320*g* for 10 minutes in a microfuge
13. The pellet was washed three times in 100µl each of sdH_2O and dried using a Speed Vac (Savant Instruments Inc.)
14. The insoluble fimbriae were mixed for a few seconds with 90% formic acid and the acid was immediately removed using a Speed Vac concentrator (Savant Instruments Inc.) by spinning for 1 hour with heating
15. The acid treated sample was resuspended in 50µl of sample buffer (refer to appendix 2) and loaded into one well of a polyacrylamide gel (12% resolving and 4% stacking).
16. 20µl of a low molecular weight protein marker (Pharmacia Biotech (Australia) Pty Ltd) was prepared as per the manufacturer's instructions and loaded onto the gel
17. Electrophoresis was performed at 50 mA for 3 hours
18. Gels were stained with Coomassie Blue using the method of Sambrook *et al.* (1989)

3.2. d (ii) Western blotting with anti-SEF17 antibody

Western blotting was performed in collaboration with Dr Karen Collinson of the Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia. The isolates tested were the same 47 isolates tested for Congo red binding by Dr Collinson (refer to section 3.2a (i)). The method used for immunoblotting was as reported by Collinson *et al.* (1991). Briefly, the isolates were grown on T medium or T medium containing congo red for periods of 24 hours and

12 days at 37°C, and 48 hours and 12 days at room temperature (ca. 21°C). Whole cells were scraped from the plates after incubation and processed for SDS-PAGE using conditions that depolymerize SEF17 to the AgfA fimbrin subunits. Proteins were separated on gels and then transferred to nitrocellulose membrane. AgfA bands were visualized by incubating blots with rabbit serum raised against SEF17 (first antibody) followed by a second antibody, goat anti-rabbit IgG, conjugated to alkaline phosphatase. Western blots were developed with BCIP and NBT to visualize immunoreactive bands.

3.3 Results

3.3. a) Phenotypic testing

3.3. a (i) Congo red binding

Isolates were tested for their ability to bind the hydrophobic dye, Congo red, as an indicator of the presence of thin aggregative fimbriae (refer to section 3.2a i). Isolates 3, 7, 12, 14, 16-20, 22, 24, 26, 29, 31, 35-42, 47-51, 53, 60, 66, 69-74, 77-79, 85, 88-91, 93-96, 99 and 101 were tested using only Method 1. All were positive for Congo red binding except isolates 19, 24, 91 and 93. *S. enteritidis* 6/E5 was also positive by Method 1. Table 3.1 is a summary of the results obtained for isolates on which both methods were used. Figure 3.1 is a photograph of Congo red agar plates demonstrating binding and non-binding isolates. For each of the five isolates there are two plate cultures, one incubated at 37°C and the other at ca. 21°C, both for 6 days.

Table 3.1 - Results of the Congo red binding assay

Isolate	Method 1	Method 2						
	37°C→21°C[a]	37°C				room temperature (ca.21°C)		
	1 day→6 days[a]	1 day	2 days	5 days	11 days	2 days	5 days	11 days
1	+	d	D	D	+++	-	-‖o	O
2	+	d	D	D	+++	-	-	O
4	+	-‖o	O‖D	D	+++	-	d	D
5	+	o	D	D	+++	-	o	D
8	+	-‖o	o‖++	o‖++	+++	-	-‖o	-‖+‖O
9	+	-‖d	+‖++	++	+++	-	-‖o	O‖D
11	+	d	D	D	+++	-	+	o
13	-	-‖o	D	D	+++	-	-	o
15	+	o‖d	D	++	+++	-	-‖+	O

Isolate	Method 1	Method 2						
	37°C→21°C[a]	37°C				room temperature (ca.21°C)		
	1 day→6 days[a]	1 day	2 days	5 days	11 days	2 days	5 days	11 days
21	-	o	D	D	+++	-	-	O
23	+	-	O	++	+++	-	-	O
25	-	-‖o	O	D	+++	-	-	O
27	+	o	D	D	+++	-	-	O
28	+	-	D‖++	D‖++	+++	-	-	-‖+
30	+	-	o	D	+++	-	-	D
33	+	d	++	++	+++	-	-‖++	++
34	+	o	D	D	+++	-	-	D
43	+	o	D‖++	D‖++	+++	-	-‖o	D
44	+	-	O‖++	++	+++	-	-	D
45	+	-	O‖D	D	+++	-	-‖o	D
46	+	o	D	++	+++	-	-	O
52	+	o	O‖++	D‖++	+++	-	-	o‖D
54	+	o	o‖++	D‖++	+++	-	-‖o	D
55	+	-‖o	o‖++	D‖++	+++	-	-	O‖++
56	-	-	o	++	+++	-	-	O‖D
57	+	d	++	++	+++	-	-	D
58	-	-	o	++	+++	-	-	O‖D
59	+	o	d	++	+++	-	-	O
61	+	o	O	++	+++	-	-	o
62	+	-‖o	O‖D	++	+++	-	-	O‖d
63	+	-‖o	O	++	+++	-	-	O
64	+	-‖o	D	++	+++	-	+‖++‖ +++	++‖+++
67	+	o‖d	D	++	+++	-	-	d
68	+	-	o‖O	++	+++	-	-	o
75	+	-	o	++	+++	-	-‖o	D
76	+	-	++	++	+++	-	-	D
80	+	o‖d	D	++	+++	-	-	D
81	+	-	O‖++	D‖++	+++	-	-	o‖D

Isolate	Method 1	Method 2						
	37°C→21°C[a]	37°C				room temperature (ca.21°C)		
	1 day→6 days[a]	1 day	2 days	5 days	11 days	2 days	5 days	11 days
82	+	o‖d	D‖++	D‖++	+++	-	-‖o	-‖d
83	+	-	+	++	+++	-	-	o
84	+	o	d	++	+++	-	-	o
86	+	-	o‖++	++	+++	-	-	++
87	+	-‖d	O‖D	++	+++	-	-	D
92	+	-	o	D	+++	-	-‖o	o
97	+	o‖d	+‖++	++	+++	-	-	D
98	+	o	D	D‖++	+++	-	-‖+	d‖D
100	-	-	O	++	+++	-	-	o‖d
SE 27655-3b [b]	NT	+++	+++	+++	+++	+++	+++	+++

[a] Conditions of growth by Method 1 - incubation at 37°C for 1 day followed by incubation at 21°C for a further 6 days.

[b] SE 27655-3b = *S. enteritidis* 27655-3b (positive control strain)

-, no binding (pink colonies)

o, scant binding (pink colonies with small central area of colony light orange)

d, scant binding (pink colonies with small central area of colony dark orange)

O, slight binding (pink colonies with large central area of colony light orange)

D, slight binding (pink colonies with large central area of colony dark orange)

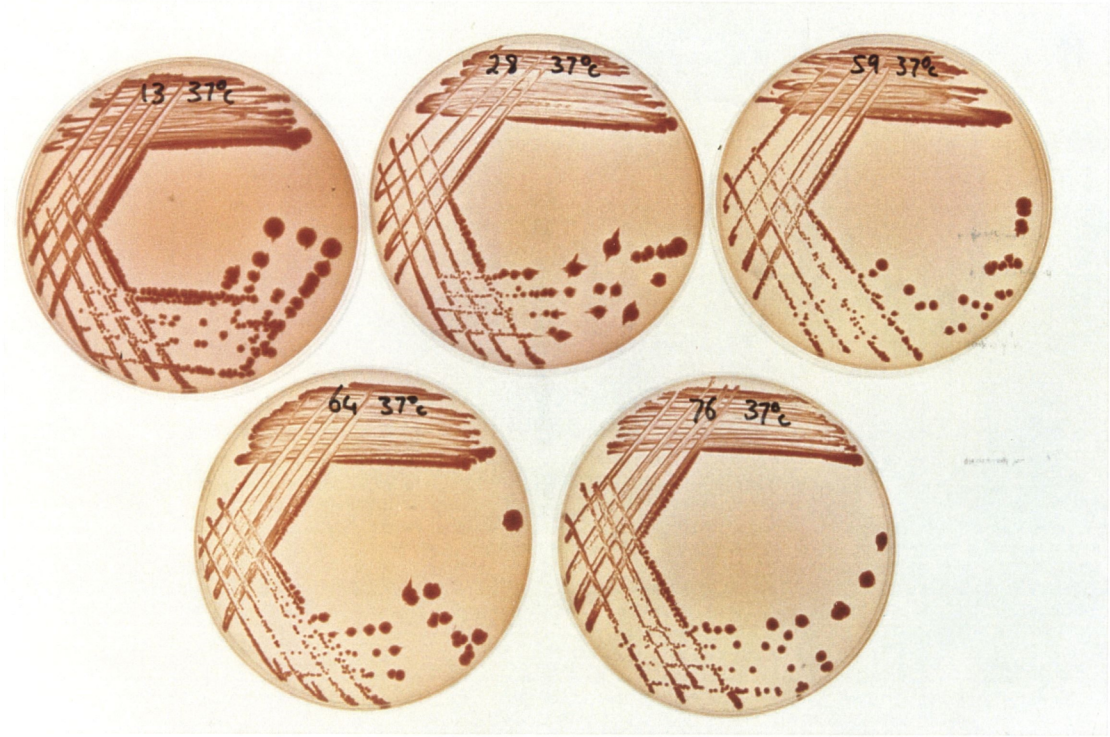
+, some binding (whole colony slightly orange)

++, moderate binding (whole colony dark orange)

+++, strong binding (whole colony red)

||, divides multiple CR binding types for a given strain

A



B

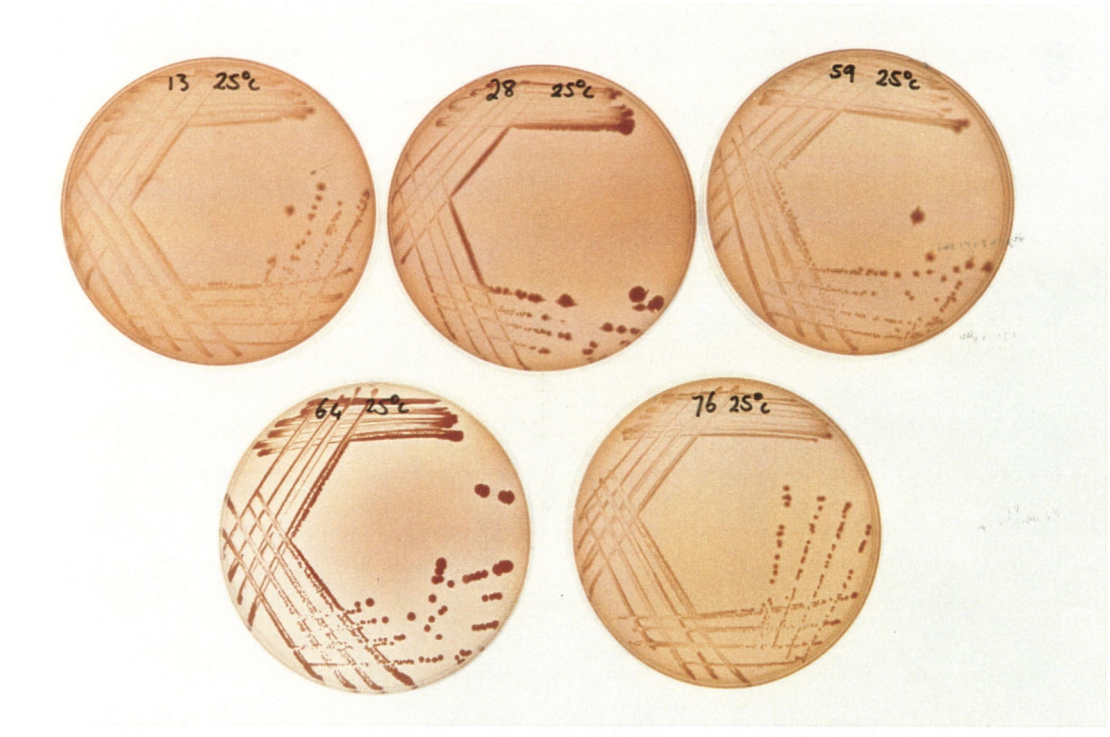


Figure 3.1- Results of the Congo red binding assay. Clockwise from top left on A and B - isolate 13, isolate 28, isolate 59, isolate 76 and isolate 64. (A) shows the isolates after growth at 37°C for 6 days and (B) shows the colonies after growth at 25°C for 6 days

3.3. a (ii) Colony morphology on T medium

All *S. virchow* isolates were examined for wrinkled colony morphology on T medium (refer to section 3.2a ii). Table 3.2 shows that 64 out of 95 (67.4%) isolates were positive for wrinkled colony morphology.

3.3. a (iii) Colony morphology on CFA agar

All *S. virchow* isolates were grown of CFA agar to examine for contoured/wrinkled colony morphology (refer to section 3.2a iii). Table 3.2 also shows that 64 out of 95 (67.4%) isolates were positive for contoured colony morphology.

3.3. a (iv) Growth in static CFA broth culture

All *S. virchow* isolates, excluding isolate 80, were grown in static CFA broths for pellicle formation (refer to section 3.2a iv). Table 3.2 shows that 68 out of 94 (72.3%) isolates were positive for pellicle formation.

Table 3.2 - Results for colony morphology on T medium and CFA agar and pellicle formation in static CFA broth cultures

Isolate	T medium[a]	CFA medium[b]	static CFA broth[c]	Isolate	T medium[a]	CFA medium[b]	static CFA broth[c]
1	+	+	+	17	-	-	+
2	+	+	-	18	+	+	-
3	+	+	+	19	+	+	-
4	+	++	+	20	-	-	+
5	++	++	+	21	-	-	-
7	+	++	+	22	-	-	+
8	+	+	-	23	+	+	-
9	-	-	-	24	-	-	-
11	+	+	+	25	-	-	-
12	-	-	-	26	+	+	+
13	-	-	+	27	+	-	-
14	-	-	-	28	++	++	+
15	+	+	+	29	-	-	-
16	-	-	-	30	+	++	+

Isolate	T medium[a]	CFA medium[b]	static CFA broth[c]
31	++	++	+
33	-	-	-
34	+	++	-
35	-	-	+
36	++	++	+
37	+	+	+
38	+	+	+
39	++	++	+
40	-	-	-
41	+	++	+
42	+	++	+
43	+	++	+
44	++	+	+
45	++	+	+
46	-	-	-
47	+	++	+
48	++	++	+
50	++	++	+
51	-	-	+
52	+	+	+
53	+	+	+
54	++	++	+
55	++	++	+
56	-	-	+
57	-	-	-
58	-	-	-
59	-	-	-
60	+	+	+
61	+	+	+
62	++	++	-
63	+	++	+
64	++	++	+
66	++	++	+
67	+	+	-
68	++	++	+
69	++	+	+
71	++	+	+
72	-	-	-
73	+	+	+
74	-	-	+
75	++	+	+
76	-	-	-
77	++	+	+
78	++	+	-
79	+	+	+
80	+	+	NT
81	++	+	+
82	++	++	+
83	-	-	+
84	-	-	+
85	++	+	+
86	++	+	+
87	++	++	+
88	++	++	+
89	++	++	+
90	+	+	+
91	+	+	+
92	-	+	+
93	-	-	+
94	-	-	+
95	++	++	+
96	++	++	+
97	+	+	+
98	++	+	+
99	+	+	+
100	-	-	+
101	-	-	+
SE 6/E5[d]	++	++	+

[a] T medium - ++, very rough colonies; +, intermediate roughness; -, smooth colonies

[b] CFA medium - ++, highly contoured colonies; +, intermediate contouring; -, smooth colonies

[c] CFA broth cultures - +, pellicle of growth on surface of broth; -, uniformly turbid broth; NT, not tested

[d] SE 27655-3b = *S. enteritidis* 27655-3b (positive control strain)

3.3. b) Molecular Detection of the *agfA* gene by PCR

A PCR protocol to amplify a region of the *agfA* gene, which encodes SEF17 fimbrial protein subunits, was applied to *S. virchow* DNA (refer to section 3.2b). Figure 3.2 shows the results of a PCR reaction for several isolates including two positive isolates and one negative isolate.

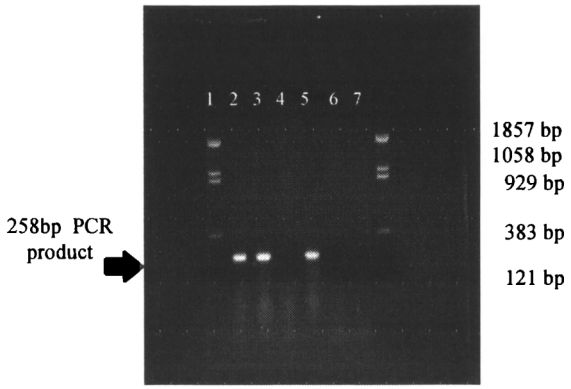


Figure 3.2 - Results of PCR detection of the *agfA* gene. Lane 1 and 8 - pBR322 DNA digested with *BstNI* marker DNA, lane 2 - isolate 98, lane 3 - isolate 99, lane 4 - isolate 100, lane 5 - isolate *S. enteritidis* 6/E5 - positive control, lane 6 - *E. coli* ACM 1803 - negative control, lane 7 - negative PCR control

Isolates 58, 59 and 100 were the only isolates which did not result in amplification of a PCR product using the reaction conditions outlined in section 3.2 b iii). To ensure these were not false negatives further testing of these isolates was done. This testing included varying template and $MgCl_2$ concentrations (refer to section 3.2 b iii). All three isolates remained negative by PCR, under all conditions tested.

3.3. c) Sequence Analysis of *S. virchow agfA* PCR product

The nucleotide sequence of the *agfA* PCR product from isolate 5 was determined and the amino acid sequence found to be identical to the corresponding region of *S. enteritidis* 27655-3b. The *agfA* nucleotide sequence of *S. virchow* is aligned to the *agfA* region of the *S. enteritidis* 27655-3b *agfBAC* operon (GenBank U43280) in Figure 3.3. Comparison of the two nucleotide sequences showed seven base substitutions over the 258bp region of the *agfA* PCR product. Two of these substitutions were in the forward primer region and were artefacts of the primer sequence used. The five remaining substitutions were in the third codon and therefore the predicted amino acid sequences were identical.

3.3. d) Expression of SEF17

3.3. d (i) Confirmation of fimbrial expression by electron microscopy

Electron microscopy was used to detect fimbriae-like structures on *S. virchow* cells (refer to section 3.2 c). Figure 3.4 is an electron micrograph of isolate 64 showing SEF17-like fimbrial structures of the expected diameter (ca. 4-5nm) (Collinson *et al.*, 1991).

3.3. d (ii) SDS-PAGE analysis of purified protein

The modification of the protein purification method (refer to section 3.2d i) resulted in a purified protein from the cells of *S. virchow* isolate 64. Polyacrylamide gel electrophoresis of the purified protein and a molecular weight marker confirmed the size of this protein as appropriate for the AgfA subunit protein. Figure 3.5 shows the purified protein from isolate 64, which is approximately 17kDa.

```
agfBAC    ATGAAACTTTTAAAAGTGGCAGCATTCGCAGCAATCGTAGTTTCTGGCAGTGCTCTGGCT
aa seq     M  K  L  L  K  V  A  A  F  A  A  I  V  V  S  G  S  A  L  A

agfbac    GGCGTCGTTCCACAATGGGGCGGCGGCGGTAATCATAACGGCGGCGGCAATAGTTCCGGC
isol 5        TCGTACCACAGTGGGGCGGCGGCGGTAATCATAATGGCGGCGGCAATAGTTCCGGC
              **** ***** ********************** **********************
aa seq     G  V  V  P  Q  W  G  G  G  G  N  H  N  G  G  G  N  S  S  G

agfbac    CCGGACTCAACGTTGAGCATTTATCAGTACGGTTCCGCTAACGCTGCGCTTGCTCTGCAA
isol 5    CCGGATTCCACGTTGAGCATTTATCAGTACGGTTCCGCTAACGCTGCGCTTGCTCTGCAA
          ***** ** **************************************************
aa seq     P  D  S  T  L  S  I  Y  Q  Y  G  S  A  N  A  A  L  A  L  Q

agfbac    AGCGATGCCCGTAAATCTGAAACGACCATTACCCAGAGCGGTTATGGTAACGGCGCCGAT
isol 5    AGCGATGCCCGTAAATCTGAAACGACTATTACCCAGAGCGGTTATGGTAACGGCGCCGAT
          ************************* **********************************
aa seq     S  D  A  R  K  S  E  T  T  I  T  Q  S  G  Y  G  N  G  A  D

agfbac    GTAGGCCAGGGTGCGGATAATAGTACTATTGAACTGACTCAGAATGGTTTCAGAAATAAT
isol 5    GTAGGCCAGGGTGCGGATAATAGTACTATTGAACTGACTCAGAATGGTTTCAGAAACAAT
          ************************************************************ ***
aa seq     V  G  Q  G  A  D  N  S  T  I  E  L  T  Q  N  G  F  R  N  N

agfbac    GCCACCATCGACCAGTGGAACGCTAAAAACTCCGATATTACTGTCGGCCAATACGGCGGT
isol 5    GCCACCATCGACCAGTGGAA
          ********************
aa seq     A  T  I  D  Q  W  N  A  K  N  S  D  I  T  V  G  Q  Y  G  G

agfbac    AATAACGCCGCGCTGGTTAATCAGACCGCATCTGATTCCAGCGTAATGGTGCGTCAGGTT
aa seq     N  N  A  A  L  V  N  Q  T  A  S  D  S  S  V  M  V  R  Q  V

agfbac    GGTTTTGGCAACAACGCCACGGCTAACCAGTATTAA
aa seq     G  F  G  N  N  A  T  A  N  Q  Y  O
```

Figure 3.3 - Alignment of the *agfA* coding region from *S. enteritidis* 27655-3b (*agfBAC*) and sequence of the *S. virchow* (isol 5) *agfA* PCR product. The predicted amino acid sequence is included and nucleotide sequence identity is denoted by *

Figure 3.4- Electron micrograph of isolate 64 after growth at 37°C for 14 days on T medium. This preparation was negatively stained with 1% ammonium molybdate and 0.1% glycerol (spreading agent) at pH 7.2. Bar = 100nm. The small arrow indicates SEF 17-like fimbrial structures and the larger arrow indicates flagella

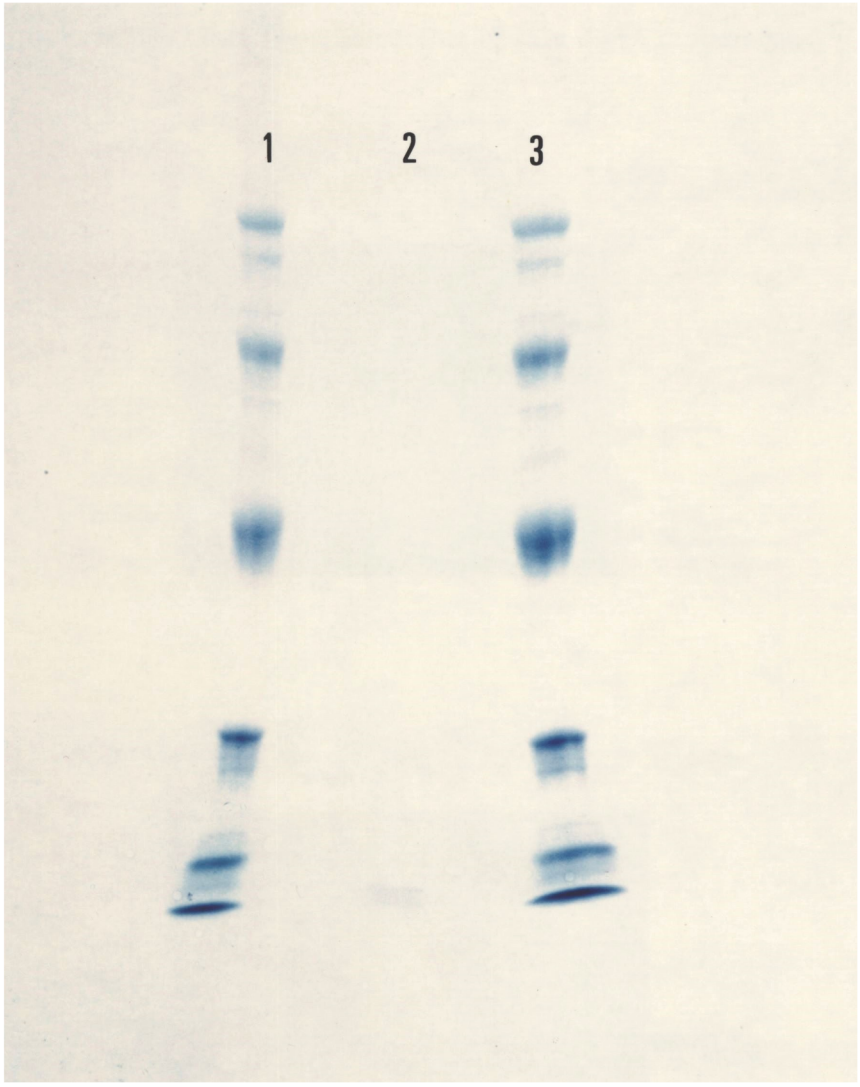


Figure 35- SDS-polyacrylamide gel showing the Coomassie blue-stained band of the purified fimbrial protein from *S. virchow* isolate 64 (Lane 2). Lanes 1 and 3 contain the protein molecular mass standard (Pharmacia Biotech (Australia) Pty Ltd) and the weights of the proteins are: phosphorylase b - 94kDa, albumin - 67kDa, ovalbumin - 43kDa, carbonic anhydrase - 30kDa, trypsin inhibitor - 21.1kDa, α-lactalbumin - 14.4kDa

3.3. d (iii) Confirmation of SEF17 protein by Immunoblotting

A group of forty-seven isolates were tested using immunoblotting, by Dr Karen Collinson (refer to section 3.2d (ii). Figure 3.6 are western blots of some of the *S. virchow* isolates and Table 3.3 presents the results of all of the *S. virchow* isolates for the presence of an immunoreactive 17 kDa AgfA protein band.

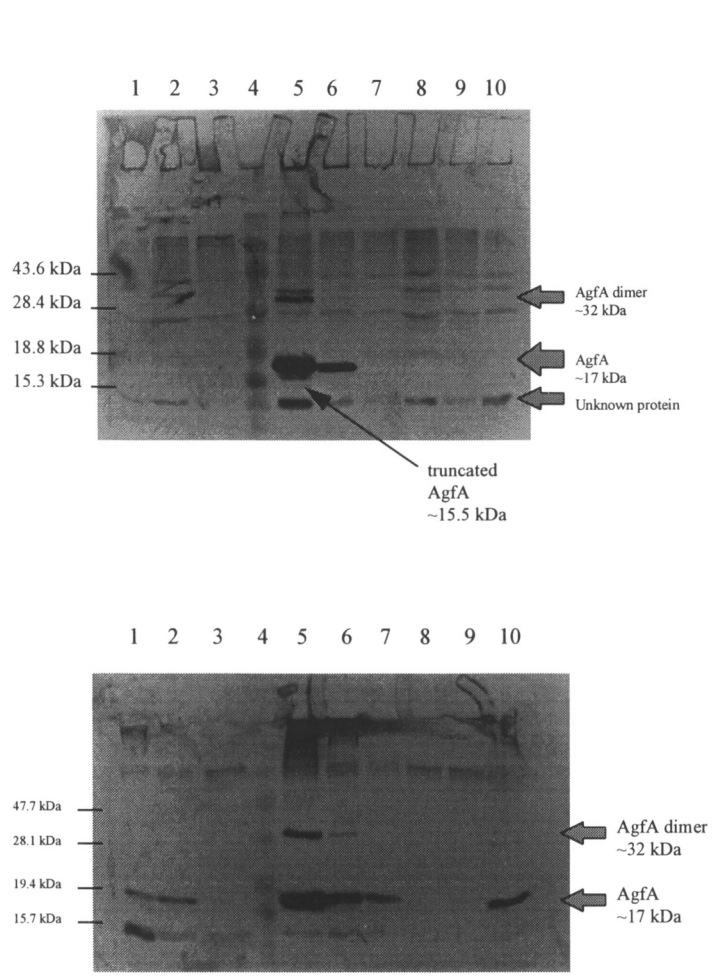


Figure 3.6 - Western blot detection of AgfA subunit protein using SEF17 antiserum. **(A)** Lane 1 - isolate 15, lane 2 - isolate 43, lane 3 - isolate 59, lane 4 - prestained protein molecular weight standard, lane 5 - *S. enteritidis* 276553b (positive control strain), lane 6 - isolate 64, lane 7 - isolate 67, lane 8 - isolate 75, lane 9 - isolate 82 and lane 10 - isolate 83. **(B)** Lane 1 - isolate 61, lane 2 - isolate 62, lane 3 - isolate 63, lane 4 - prestained molecular weight standard, lane 5 - isolate 64, lane 6 - isolate 67, lane 7 - isolate 68, lane 8 - isolate 76, lane 9 - isolate 80 and lane 10 - isolate 81

Table 3.3 - Results of detection of the AgfA fimbrin using immunoblotting

Isolate	Western blot analysis of cells grown on T or TCR			
	37°C 24h[a]	37°C 12d[a]	RT 48h[a]	RT 12d[a]
S. enteritidis 27655-3b[b]	++	++	++	++
1	-	-	+	+
2	-	-	+	++
4	-	-	++	++
5	-	+	+	++
8	-	-	-	++
9	-	-	+	++
11	-	-	+	++
13	-	-	-	-
15	-	+	-	++
21	-	-	-	-
23	-	-	-	-
25	-	-	++	+
27	-	-	+	+
28	-	-	-	++
30	-	-	+	++
33	-	-tr	++	++
34	-	-	++	+
43	-	-	++	++
44	-	-	+	++
45	-	-	-	++
46	-	-	-	-
52	-	-	-	++
54	-	-	+	++

Isolate	Western blot analysis of cells grown on T or TCR			
	37°C 24h[a]	37°C 12d[a]	RT 48h[a]	RT 12d[a]
55	-	-	-	++
56	-*	-*	-*	-*
57	-	-	-	-
58	-*	-*	-*	-*
59	-	-	+	+
61	-	-	+	+
62	-	-	+	+
63	-	-	-	-
64	++	++	++	++
67	-	-	+	++
68	-	-	-	++
75	-	-	-	++
76	-	-	-	-
80	-	-	-	-
81	-	-	+	++
82	-	-	+	++
83	-	-	+	++
84	-	-	-	-
86	-	-	-	++
87	-	-	-	-
92	-	-tr	-	++
97	-	-	+	-
98	+	-	++	++
100	-*	-*	-*	-*

[a] Conditions for growth of isolates

[b] SE 27655-3b = *S. enteritidis* 27655-3b (positive control strain)

TCR, T medium containing congo red at 100µg/ml

++, very strong

+, moderate to weak band

-, no detectable band

-tr, normally negative but some samples taken from portions of the plate with some Congo red binding colonies were faintly positive

-*, very faint band migrating slightly higher than AgfA but probably not AgfA

3.4 Discussion

agfA is the gene which encodes the AgfA 17kDa structural subunit of SEF17 fimbrin. The *agfA* gene has been shown to be widely distributed in 95 serovars of the genus *Salmonella* (Doran *et al.*, 1993) however, *S. virchow* was not one of the serovars investigated.

SEF17 have been associated with the binding of tissue matrix proteins such as fibronectin, laminin and various collagen types (other than type II) by *S. enteritidis* 27655-3b (Collinson *et al.*, 1993). Interactions such as these between the host basement membrane, matrix proteins and *S. enteritidis* could provide this organism with invasive properties important for virulence. Sjobring *et al.* (1994) found that both *S. enteritidis* 27665-3b and *E. coli* which express a similar structure to SEF17 called curli, are able to bind plasminogen and the tissue-type plasminogen activator (t-PA). Plasminogen is the precursor to the fibrin-degrading protease, plasmin, and activation of plasminogen is by t-PA. This activation can occur if both plasminogen and t-PA are bound by thin aggregative fimbriae or curli. Plasmin is also able to cleave other tissue components such as collagen, fibronectin and laminin (Sjöbring *et al.*, 1994). Therefore the ability to bind plasminogen and t-PA confers a tissue-degrading capability which may play a role in these organism's capacity to penetrate deep tissue and cause invasive infection. *S. virchow* are reported to have a propensity to cause extra-intestinal infections (Ingram & Redding, 1988; Ashdown & Ryan, 1990; Ward *et al.*, 1990; Sechter *et al.*, 1991; Threlfall *et al.*, 1992; Frost *et al.*, 1996) and therefore it is important to determine if *S. virchow* possesses SEF17 because this surface structure may contribute significantly to the virulence of *S. virchow*.

The present study began by screening the 95 *S. virchow* isolates for fimbrial expression. Investigation of the congo red binding ability of the isolates was performed by two methods (refer to section 3.3a i). Method 1 was a relatively rapid test and method 2 was a time course study to obtain a more detailed insight into the binding capacity of the *S. virchow* isolates. Not all the isolates were tested by method 2.

Method 1 used a combined incubation regime involving incubation at 37°C followed by incubation at 21°C. It was considered that an ideal screening method would identify the maximum number of positive isolates, and for this reason a combined incubation was used. Temperature had been shown to be an important factor in the expression of surface structures (Arnqvist *et al.*, 1992; Iriarte & Cornelis, 1995). The gene, *csgA*, which encodes curli of *E. coli* has been shown to be transcriptionally activated on CFA medium, at 26°C but not at 37°C (Arnqvist *et al.*, 1992). In contrast, the *myfA* gene which encodes the 21kDa subunit protein of Myf fibrillae of *Yersinia enterocolitica* is transcribed only at 37°C (Iriarte & Cornelis, 1995). It is also suspected that cycling of incubation temperatures may induce strong synthesis of SEF17 by *S. enteritidis* (pers. comm. - Dr J.M. Cox) and so changing the temperature during incubation may be an effective method to promote fimbrial expression.

When the results of methods 1 and 2 were compared, method 1 identified all but six of the isolates that were positive by method 2. Three of the exceptions, isolates 13, 21 and 25, were shown to bind congo red strongly only after 11 days at 37°C and slightly after 11 days at 21°C. Method 1 would not identify these isolates because the total incubation time was only 7 days. The other three exceptions were isolates 56, 58 and 100, all of which were positive after incubation at 37°C for five days. Again, method 1 would not identify these three isolates because the 37°C incubation component of method one was only one day. The results of this comparison suggested that method 1 was a reasonable screening method for congo red binding.

Method 2 demonstrated the different levels of congo red binding that occurred and the effects of incubation time and temperature. The *S. virchow* isolates bound congo red weakly in comparison with the positive control strain of *S. enteritidis* 27655-3b. None of the *S. virchow* isolates bound congo red after 24 hours at 37°C with the exception of isolate 64 which was the strongest *S. virchow* producer but it was not as strong as *S. enteritidis* 27655-3b. The strongest binding was seen after 11 days at 37°C. In addition, many of the isolates bound congo red after incubation at 21°C, but only after 5 to 11 days.

A major difficulty of the congo red binding was the scoring of positive binding. There were many degrees of positive binding, ranging from small orange areas of colonies to completely dark red colonies. This introduced subjectivity which would make it very difficult to compare results performed by different groups.

There are many factors which can influence the binding of congo red. Incubation time and temperature were demonstrated in this study. Another important influence not investigated in this study was media. Eglezos (1994) found there was good correlation between colony morphology and virulence using CFA agar and CFA agar containing congo red. Nutrient availability or the distribution of the dye within the agar may also play a role in the colony morphology. Colonies of the same isolate, in different areas of a plate, were observed to bind different amounts of congo red. Therefore it is difficult to determine whether this is a difference in expression of fimbriae by different cells of the same strain or an artefact of the media used.

A limitation of congo red binding as a screening method for SEF17 is that the dye is not exclusively bound by SEF17. Genes have been identified in *Shigella flexneri* which encode a protein moiety expressed on the surface of cells that binds congo red. These genes are located on the chromosome of *Shigella flexneri* as well as the 230kb virulence plasmid of this serovar (Sakai *et al.*, 1986). In *Aeromonas salmonicida*, congo red dye is bound by the A-protein component of the surface protein array called the A-layer (Ishiguro *et al.*, 1985). Since congo red binding is not exclusively due to the presence of SEF17 and therefore not a definitive indicator of SEF17 expression, a DNA-based method was also applied. This method identified *S. virchow* isolates which possessed the gene that encodes the subunit protein of SEF17, AgfA.

To determine if the congo red binding was an adequate indicator of SEF17 production, comparison of the congo red binding results from method 2 and the immunoblotting with SEF17 specific antiserum was performed. This comparison highlighted some inconsistencies, in particular false positive and false negative results. A false positive result was defined as congo red binding positive but SEF17 negative by immunoblotting. Examples of this were the isolates which were negative by Western blot analysis at both 37°C and 21°C (isolates 13, 21, 23, 46, 56, 57, 58, 63, 76, 80, 84, and 100). All of these isolates were strongly positive (+++) for congo

red binding after 11 days at 37°C. Despite the colonies red appearance, very few produced SEF17. There could be several explanations for this, including the production of SEF17 protein being too low to be detected, or perhaps the binding of congo red by other mechanisms in these cells. As mentioned previously, congo red binding proteins located on the cell surface have been described for *Shigella* spp., and *Aeromonas salmonicida* and it is likely that *Salmonella* also have these proteins which bind congo red (Ishiguro *et al.*, 1985; Sakai *et al.*, 1986).

A false negative result was defined as negative congo red binding but Western blots which detected the SEF17 protein. An example was isolate 4 which demonstrated no congo red binding after growth at 21°C for two days but had a strong SEF17 band on Western blots. There were many other examples of isolates grown at room temperature which demonstrated SEF17 protein by immunoblotting regardless of whether the binding of congo red was scant, slight or strong (Dr K. Collinson - pers. comm.). This suggested that the use of congo red binding to predict the expression of SEF17 was only reliable for detecting isolates which were copious producers of SEF17 such as *S. enteritidis* 27655-3b and *S. virchow* 64.

Another indicator of the expression of thin, aggregative fimbriae is colony morphology on T medium. *S. enteritidis* 27655-3b grown on T medium produces aggregative rough colonies which adhere to the agar. Bacterial cells from these colonies autoaggregate and are difficult to resuspend (Collinson *et al.*, 1991; Müller *et al.*, 1991). Of the 95 *S. virchow* isolates tested, 64 grew as rough colonies on T medium. However *S. enteritidis* 27655-3b was not available for inclusion and so direct comparison of positive results on T medium was not possible. It was understood from the results of the congo red binding, Western blotting and observations by Dr K Collinson that none of the *S. virchow* strains had the degree of roughness that the *S. enteritidis* strain had. However, the correlation between T medium morphology and detection of SEF17 by immunoblotting was good. Of the twelve isolates which were negative for SEF17 by Western blotting, nine were also recorded as mucoid (non aggregative) colonies on T medium. The other three isolates that were negative by Western blotting (isolates 23, 63 and 80) were recorded as having weak to intermediate roughness on T medium.

It has been suggested that the wrinkled appearance on T medium is due to the aggregative nature of the SEF17 fimbriae, causing the cells to be pulled together as they multiply to form colonies (pers. comm. - Dr J.M. Cox). If the colony morphology on this medium is exclusively due to the expression of SEF17 it is likely that the degree of wrinkling is directly proportional to the amount of fimbriae expressed. Therefore cells which are prolific producers of SEF17 will be more readily detected by this method.

The growth of colonies on CFA agar uses the same indicators to identify expression as T medium. A wrinkled or contoured morphology is indicative of cells expressing fimbriae and the more prolific the expression the more contoured the colonies appear. There was very strong correlation between the results of T medium and CFA agar. Only isolates 27 and 92 exhibited disparate results with isolate 27 positive by T medium and not CFA and the opposite result for isolate 92.

Static CFA broths was the fourth predictive test for the expression of aggregative fimbriae. As with the other phenotypic tests, the results of the *S. virchow* isolates in static CFA broths were not as convincing as *S. enteritidis* 27655-3b. *S. enteritidis* 27655-3b produces a surface pellicle after growth for 48 to 72 hours (Collinson *et al.*, 1993). Pellicle formation was not recorded for any *S. virchow* isolates in the first 48 hours of incubation at 37°C and for this reason the broth cultures were incubated longer.

Very little detail of how to perform this test was published, especially with respect to the volume of broth used and temperature of incubation. For this reason some preliminary work involved repeating the method using different equipment such as beakers, flasks and test tubes. Test tubes were finally chosen because the surface area of the broth had a large effect on whether a pellicle would form. The surface area of broth was too large when flasks and beakers were used and pellicles did not form readily.

Media components were also found to alter the results of static CFA broth testing. A change in the supplier of casamino acids lead to changes in the results

obtained for several of the isolates. Therefore, the entire collection was retested on the same batch of media, using casein hydrolysate (Oxoid Australia Pty. Ltd.).

Incubation times also had a significant effect on the results. Other examples of the effect of incubation time have been reported in the literature. Transcriptional activation of the curli gene, *csgA,* in *E. coli* HB101 does not occur while cells are growing exponentially in CFA broth but the gene is transcribed after 48 hours, when the cells have reached stationary phase (Arnqvist *et al.*, 1992). It would seem that a similar effect existed for *S. virchow* isolates. Pellicle formation was not recorded for any *S. virchow* isolates in the first 48 hours of incubation.

These phenotypic methods employed both solid and liquid media but direct comparison of these results should be done with caution. Collinson *et al.* (1991) reported that *S. enteritidis* 27655-3b SEF17 are produced on both solid and liquid media. However, differences in results when both solid and liquid media are used may not be due solely to differences in fimbrial expression but may simply be due to the cells growing differently in liquid versus solid media. Liquid medium and solid medium may also have different levels of sensitivity to detecting fimbrial expression. For this reason it is best to compare solid phase tests with each other. When this was done in the present study very strong correlation between T medium and CFA agar was seen, with only two isolates out of ninety-five in disagreement. By comparison less correlation was observed between tests of different medium phases. There were 25 isolates out of ninety-four for which disparate results were seen when both of the agar methods were compared to the static broth test. These contradictions may reflect differences due to the cell's growth conditions, rather than true differences in fimbrial expression. Further testing should involve growth on media prepared in both solid and liquid phase for accurate comparison.

Since the expression of SEF17 has been shown to be highly variable between serovars (Doran *et al.*, 1993) and within serovars (current study), a genotypic method to detect the gene which encodes the SEF17 structural protein was developed to screen *S. virchow* isolates. The genotypic method to detect this gene was by PCR. The PCR primers were designed from the only SEF17 sequence available at the time,

which was for the *agfA* gene from *S. enteritidis* 27655-3b (GenBank accession number S65743) (Doran *et al.*, 1993).

The PCR of *S. virchow* isolates determined that 96.8% (92/95) of the isolates encoded *agfA* since a single PCR product of the expected size was amplified. Only three isolates were negative by PCR for *agfA*, isolates 58, 59 and 100. However, the result for isolate 59 was shown to be irregular because this isolate tested positive by Western blotting, indicating that it does produce AgfA fimbrin and therefore has the *agfA* gene. A possible reason why this isolate did not have a positive PCR result is discussed later. Both of the other PCR negative isolates were also negative by Western blotting which supported the result that they lack the *agfA* gene and do not express SEF17.

Direct sequencing of the product from isolate 5 was performed to determine the nucleotide sequence of the amplified region of the *agfA* gene. Although a molecular probe has been used to detect the *agfA* gene in numerous *Salmonella* serovars, sequence data is only available for one strain, *S. enteritidis* 27655-3b. There is no sequence data for *S. virchow* and therefore this was a novel contribution to the knowledge of the prevalence of *agfA* and its sequence conservation in *Salmonella* spp. The nucleotide sequence of the PCR product between the primers differed by only five bases when compared with the nucleotide sequence of *S. enteritidis* 27655-3b. Furthermore, all of these base changes were in the third base of a codon and therefore no change in the amino acid occurred. In fact the amino acid sequence was identical for *S. virchow* and *S. enteritidis* in the region under investigation suggesting that *agfA* is a highly conserved gene between serovars and that the fimbrin subunit protein has a functional importance.

The *agfA* gene sequence from which the PCR primers were designed in this study was superseded in 1996 by the full sequence of the *agfBAC* operon (Collinson *et al.*, 1996a). This operon includes the *agfA* gene which is transcribed with the *agfB* gene for which, at this time, there is no known function. A third open reading frame, *agfC*, is also included in this operon. Comparison of the original sequence data of the 333bp fragment of the *agfA* gene (GenBank S65743) and the most current sequence of the *agfBAC* operon (GenBank U43280) including the entire *agfA* gene, showed that several bases of the sequence in the *agfA* coding region had been

changed. Two of these changes in particular had a significant bearing on the results of the sequencing of *S. virchow*. Using the nucleotide numbering of *agfBAC*, the region of *agfA* chosen as the forward primer in this study was nucleotides 1256 to 1273. Within this 18bp region there have been two changes in the published sequence data. Diagrammatically represented below, is the sequence of the forward primer (Agff) that was designed from the original *agf*A sequence and the most recently published sequence data for the same region in the *agfBAC* operon (*S. enteritidis* sequence).

Agff	5' GTCGTACCACA**G**TGGGGC 3'
S. enteritidis sequence	5' GTCGT**T**CCACA**A**TGGGGC 3'

The consequence of these changes was that the PCR products of *S. virchow* isolates amplified with the Agff primer contain the bases corresponding to the sequence of the primer. Therefore these will appear as base substitutions when those sequences are compared to the *agfBAC* sequence. However, the differences are an artefact of the primer sequence and do not reflect true dissimilarity between the *S. virchow* and *S. enteritidis* strains. To confirm this a forward primer upstream of this region should be employed to generate a PCR product which is sequenced.

Although these base substitutions are not at the 3' end of the Agff primer it may have been a contributing factor to the inability to obtain a PCR product for isolate 59. If this isolate had other sequence dissimilarities in the forward primer region, the increased instability due to the two introduced mismatches may have been sufficient to render the primer unable to anneal at the annealing temperature of 60°C used. Some optimization was attempted by altering the DNA template and $MgCl_2$ concentrations. However, this optimization should have included the use of lower annealing temperatures to reduce the stringency of the annealing conditions, as less stringent conditions may have allowed the non-optimal primer to anneal.

Further work should aim to determine the reasons for the failure of PCR to detect *agfA* in isolate 59. Altering annealing temperatures using the current primers and testing a new primer that does not contain the two mismatches, would be

appropriate starting points. Another approach could be to label the *agfA* PCR product and use it to probe DNA of isolate 59 using low stringency hybridizations.

In the region between the two primers used to generate the *S. virchow agfA* sequence there were five other nucleotide substitutions between the *S. virchow* isolate 5 and *S. enteritidis* 27655-3b sequences. However, all of these substitutions were in the third position of the codon so when the two nucleotide sequences were translated into predicted amino acid sequences there was 100% identity. This predicted protein sequence allowed confirmation that the thin aggregative fimbriae of *S. virchow* possess the conserved N-terminal amino acid sequence which defines the GVVPQ fimbriae (Collinson *et al.*, 1992). GVVPQ is the name that was given to thin aggregative fimbriae found in *S. enteritidis* encoded by *agfA* and in *E. coli* encoded by *csgA.* (Collinson *et al.*, 1992). Further sequencing of the entire *agfA* gene and the flanking regions, including *agfB* and *agfC* in the *agfBAC* operon of *S. virchow*, would be valuable to gain some insight into the homology of these genes between strains from different serovars and the functionality of the gene products. These findings could have significance in understanding the pathogenicity of *S. virchow.*

After considering the PCR and sequencing results it was clear that *S. virchow* isolates possessed the gene to produce SEF17. The next stage was to use electron microscopy (EM) and negative staining to visualize cells with thin, aggregative fimbriae. The Western blotting was done concurrently and indications were that isolate 64 was the strongest producer. This was supported by the congo red binding results and so this isolate was chosen for electron microscopy.

Initially, different negative stains were tested including 1% and 2% ammonium molybdate and bacitracin (spreading agent) at pH 6.8, 1% uranyl acetate plus sucrose and phosphotungstic acid. The best contrast between the cells and the background, which allowed the fimbriae to be visualized was achieved using 1 % ammonium molybdate. Various types of grids were also trialed including formvar, carbon coated and UV-treated carbon coated (hydrophilic). It was not possible to use an overnight 37°C plate culture as is done with *S. enteritidis* 27655-3b because the *S.*

virchow isolates do not produce enough fimbriae under these conditions. After incubation for 14 days the bacterial cells were put onto the grids by two methods. Floating the grid on a drop of a heavy cell suspension was sometimes sufficient, while at other times the drop of cell suspension had to be placed on top of the grid and left for the cells to attach. The length of time necessary for staining was also variable, from 30 seconds to two minutes. Thin fimbriae of ca. 3-5nm in width were observed on cells and were located all over the cell surface.

The most difficult and frustrating aspect of the microscopy was the very low proportion of cells that were producing fimbriae at any given time. Often the cells that had fimbriae were clustered together in one area of the grid, perhaps not unexpectedly due to the aggregative nature of the fimbriae. This may not be such a hindrance for strains which are stronger producers of SEF17 than *S. virchow* isolate 64 from this study. However the observation by electron microscopy of a low proportion of cells producing fimbriae does correlate with the non-uniform binding of congo red observed. This correlation suggests that the different binding of congo red is due to differences in expression of fimbriae by different cells of the same strain rather than an artefact of the medium used or distribution of the dye in the medium.

After thin, flexible fimbriae of approximately the expected dimensions for SEF17 were visualized on individual cells by electron microscopy, purification of the fimbrial protein was necessary to confirm that it was the correct size for the 17kDa subunit protein, AgfA. Protein purification was performed on isolate 64. The methodology used to purify SEF17 was laborious because of the need to use preparative polyacrylamide gels and retrieve the white flocculent insoluble cell material from the wells of these gels. Often much of the insoluble cell material could not be retrieved. Loading of the formic acid treated samples resuspended in sample buffer also proved problematic and often not all of the sample was loaded. Despite the technical difficulties, isolate 64 was shown to produce a protein band of ca. 17kDa by Coomassie blue staining.

The final objective for this study was to confirm that the 17kDa protein seen on the polyacrylamide gels was SEF17. This objective was performed by Western blotting with specific antiserum to purified SEF17. Thirty-four of the forty-seven

isolates tested by immunoblotting were positive for a protein of ca. 17kDa which was immunoreactive to the SEF17 specific antiserum. Of these, 88.2% produced SEF17 only when incubated at 21°C either for 48 hours, 12 days or both. Only four of the forty-seven isolates (5, 15, 64 and 98) produced an immunoreactive protein after incubation at 37°C. Isolate 64 was by far the strongest producer and it was the only *S. virchow* isolate to approach the levels of SEF17 production by *S. enteritidis* 27655-3b. This *S. virchow* isolate originated from the faeces of a domestic kangaroo and why it should be such a prolific SEF17 producer is unknown.

Coomassie blue staining is not a very sensitive detection method and did not enhance visualization of weak bands on the polyacrylamide gels. In contrast, the more sensitive detection by Western blotting detected lower levels of the 17kDa fimbrin. However, it is possible that isolates could produce amounts of SEF17 lower than the limit of detection of immunoblotting (Dr K. Collinson - pers. comm.). This limitation was demonstrated by isolates 97 and 98 for which there was SEF17 production detected at the earlier time point but not at the later. In both cases the positive result at the earlier time point was weak and possibly close to the limit of detection (Dr K. Collinson - pers. comm.). It is likely that these two isolates, and others, produce trace amounts of SEF17 which may not be detected consistently, if at all, by Coomassie blue staining or immunoblotting.

Further to the Western blotting of the *S. virchow* isolates, a notable observation made by Dr K Collinson (pers. comm.) was that there were several minor bands in the *S. virchow* samples which reacted with non - specific antibodies to Enterobacterial antigens, present in both the pre-immune rabbit serum and the serum collected after immunizing with purified SEF17. For all but three of the forty seven isolates tested the pattern of these bands was the same. However, isolates 56, 58, and 100 gave a slightly different pattern (data not shown) indicating that these three *S. virchow* isolates are different to the others. These three isolates were all negative for AgfA by Western blot but it was noted, they had a very faint band migrating slightly higher than AgfA on the gels. The suggestion that these three isolates are different to the other *S. virchow* was supported by the epidemiological typing results in which these three isolates were the only representative of ribotype 4 (refer to chapter 2). Future work aimed at identifying this larger protein which cross reacts with SEF17

antiserum could involve studies into the *agfB* and *agfC* genes. Perhaps the larger band is the product of one of these genes rather than *agfA*. It might also be possible that the transcription of the *agfBAC* operon in these isolates results in a larger protein due to base substitutions which eliminate stop codons between *agfB, agfA* and *agfC*.

The original basis for this section of the study was the hypothesis that, *S. virchow* have SEF17 fimbriae and that these fimbriae have a role in the ability of this serovar to cause invasive illnesses. The present study constitutes a preliminary investigation and addresses the first aspect of the hypothesis. The results prove that *S. virchow* possesses the gene, *agfA,* necessary to produce the SEF17 fimbrin structural subunit, AgfA. Furthermore, fimbriae-like structures were visualized on cells of a *S. virchow* isolate and the AgfA fimbrin protein from this isolate was purified and visualized on polyacrylamide gels. In addition, Dr K. Collinson demonstrated that the purified protein from this isolate, and others in the *S. virchow* group, react with SEF17 specific immune serum confirming that the *S. virchow* fimbriae are antigenically the same as the SEF17 fimbriae of *S. enteritidis* 27655-3b. Finally, approximately half of the full *agfA* gene was sequenced from a *S. virchow* isolate and shown to have 100% homology, at the amino acid level, to the translation of the same gene in *S. enteritidis*. These results and the conserved nature of this gene form a strong foundation on which to base further research to confirm the second aspect of the hypothesis; a role for SEF17 fimbriae in the pathogenesis of *S. virchow*.

The first objective of future research in confirming the role of SEF17 would be to investigate the control of fimbrial expression. It is clear from this study that there is considerable variation in the amount of fimbriae produced both within a population of cells and by individual cells. Moreover, is the fact that *S. enteritidis* 27655-3b is a more prolific producer of SEF17 than *S. virchow* strains indicative of their respective pathogenic capabilities? Molecular techniques could be used to knock out genes or introduce specific mutations in the *agfA* gene and promoter sites, and an invasion assay such as the HEp-2 cell culture invasion assay, described in Chapter 5, could be employed to show how these changes effect the bacteria's ability to invade eucaryotic cells. Results from these experiments would demonstrate whether SEF17 fimbriae are involved in invasion of eucaryotic cells and help in the

understanding of the behaviour of bacterial cells *in vivo*. However, it must be appreciated that *in vitro* studies may not accurately reflect how bacteria behave in the host environment.

The second objective could aim to determine how the SEF17 fimbriae mediate binding and invasion of host cells by *S. virchow*. Two of the roles in virulence which have been postulated for SEF17 in *S. enteritidis* are mediating attachment of the bacterial cells to the gut epithelium by binding tissue matrix proteins and assisting the bacteria to invade host tissue using plasminogen activation. It would be significant to demonstrate that *S. virchow* fimbriae also have fibronectin, plasminogen and tissue-type plasminogen activator binding capacity. These findings would advance the understanding of the potential role of SEF17 in the pathogenicity of *S. virchow*.

CHAPTER 4

INVESTIGATION OF THE LIPOPOLYSACCHARIDE STRUCTURE OF *S. VIRCHOW*

Chapter 4 : Investigation of the Lipopolysaccharide Structure of *S. virchow*

4.1 Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram negative bacteria. It is composed of three regions; the lipid A which acts to anchor the LPS in the outer membrane, and the R-core and the O-antigen which extend outward from the cell surface (McConnell & Wright, 1979). LPS acts as a receptor for bacteriophages, prevents complement activation and phagocytosis by macrophages and can induce endotoxic shock (Morrison & Ryan, 1992).

The LPS molecule is also important for its role in the typing of *Salmonella*. It is the structure and antigenic properties of the O-antigen of LPS which is the basis of serotyping, a method by which *Salmonella* can be distinguished beyond the genus level. Serogroups are determined by the O-antigen, with each serogroup having a group-specific O-antigen. Within each O group the organisms are further distinguished into serovars based on their flagellar (H) and capsular (Vi) antigens (Clarke & Gyles, 1986).

Virulence of many *Salmonella* serovars has been linked to expression of LPS with long chain O-antigen (Stocker & Mäkelä, 1971; Chart *et al.*, 1991a, 1993; Cox & Woolcock, 1994). The expression of either smooth (wild type) or rough LPS has also been correlated with phage types of *S. enteritidis*.

The hypothesis under investigation in this section was that virulent and non-virulent *S. virchow* isolates could be differentiated by their LPS structures. The aim was to determine the LPS types present in the ninety-five *S. virchow* isolates and establish if there was a correlation between the LPS type and the virulence of each strain. The virulence of each strain was predicted based on its site of isolation. Invasive isolates were anticipated to be virulent and included isolates from extra-intestinal sites such as blood or urine. The isolates from non-invasive sites were expected to be avirulent and were isolates from faeces and other non-clinical sites such as sewage effluent.

4.2 Materials and Methods

4.2. a) Investigation of *S. virchow* laboratory cultures

4.2. a (i) LPS sample preparation

A loopful of growth from each of the slope cultures of ninety-five *S. virchow* isolates (refer to Table 2.1) was used to patch inoculate a TSA plate for LPS sample preparation. The TSA plates were incubated at 37°C for 22 hours and always in the same location in the incubator to ensure the same incubation conditions. The LPS samples were prepared by the Proteinase K digestion method described by Chart *et al.* (1991a). 20µl of the LPS samples were loaded onto polyacrylamide gels and the remainder of each sample was stored at -20°C. LPS samples from two control strains were also prepared. *S. enteritidis* ACM 3696, phage type 4 was the smooth, wild-type LPS control, which had a laddered appearance and *S. enteritidis* ACM 3702, phage type 7 was the rough, LPS control, with no O-side chains and therefore shows no laddering.

4.2. a (ii) Polyacrylamide gel electrophoresis

Polyacrylamide gels were prepared and electrophoresed according to the method of Laemmli (1970) using a stock acrylamide/bis-acrylamide solution stored at 4°C (refer to Appendix 2). Stacking gels of 4% and 12% resolving gels were used. Electrophoresis was performed in 1 x running (glycine) buffer (refer to Appendix 2) at 25mA per gel for 3 hours at 4°C, using the Protean II xi Vertical Electrophoresis cell (Bio Rad Laboratories, Inc.). All samples were electrophoresed at least twice and the control strains were included on every gel.

4.2. a (iii) Silver staining

After electrophoresis the gels were silver stained by the method of Fomsgaard *et al.* (1990) with one modification. The silver staining step was incubated for 15 minutes rather than 10 minutes.

4.2. b) Investigation of variation in LPS structure

4.2. b (i) Age of cultures

A subgroup of isolates was chosen to investigate the variation in LPS structure that was observed in the initial analysis (section 4.2 a). The subgroup consisted of current laboratory cultures of the selected isolates as well as freeze-dried ampoules and glycerol stocks for some of the isolates (refer to Table 4.1). The freeze-dried and glycerol stocks represented these isolates at earlier stages of subculture. Six *S. virchow* isolates freshly isolated from chicken faeces, which had undergone minimal subculture, were also included in the subgroup. Samples were made from the growth of each culture as outlined in section 4.2 a. Table 4.1 lists the cultures of the subgroup, indicates the number of subcultures and the LPS type that each strain had been assigned in section 4.2 a.

4.2. b (ii) Incubation time

The isolates under investigation (refer to Table 4.1) were patched onto two TSA plates and incubated at 37°C for either 24 hours or 48 hours. Samples were then prepared and electrophoresed as described in section 4.2 a.

4.2. b (iii) Standardization of polyacrylamide gels

Standardization of polyacrylamide gels was attempted by the preparation of one supply of stock acrylamide/bis-acrylamide (to ensure a consistent percentage of

crosslinker) stored at 4°C. The laddered and non-laddered control strains of *S. enteritidis* 3696 and *S. enteritidis* 3702 respectively, were included on every gel.

Table 4.1 - The selected *S. virchow* isolates used to investigate LPS variation

Isolate	Source	Culture type and Subculture No.			LPS type in previous testing
		Glycerol	Freeze-dried	Lab slope	
2	frog's legs	3	4	12	B
7	human urine/faeces?	3	4	12	B
8	chicken meat	-	4	12	C
12	chicken meat	3	4	12	B
28	chicken meat	3	4	12	C
33	chicken faeces	3	-	12	B
38	human faeces	-	4	12	B
39	human faeces	4	4	12	A
40	human faeces	5	4	12	A
41	chicken meat	4	4	12	A
43	sewage effluent	4	4	12	A
44	sewage effluent	-	4	12	A
46	sewage effluent	4	4	12	B
48	chicken faeces	4	4	12	C
63	wallaby liver	-	3	9	A
A22	chicken faeces	-	-	1	NT
A23	chicken faeces	-	-	1	NT
A24	chicken faeces	-	-	1	NT
RP34	chicken faeces	-	-	1	NT
RP570	chicken faeces	-	-	1	NT
RP694	chicken faeces	-	-	1	NT

Key :

-, not applicable

NT - not tested previously

Subculture No. 1 - isolate that had been subcultured once

Subculture No. 3 - isolate that had been sucultured on three occasions

Subculture No. 4 - isolate that had been sucultured on four occasions

Subculture No. 5 - isolate that had been sucultured on five occasions

Subculture No. 9 - isolate that had been sucultured on nine occasions

Subculture No. 12 - isolate that had been sucultured on twelve occasions

4.3 Results

4.3. a) Investigation of *S. virchow* laboratory cultures

The LPS profiles of ninety-five *S. virchow* isolates were determined (refer to Section 4.2 a). All of the *S. virchow* isolates demonstrated smooth type LPS with the laddered appearance. However, three types were identified by subtle differences in the appearance of the smooth LPS, and the three types were designated types A, B and C. The differences between types A, B and C related to the appearance of the banding pattern that results from the presence of the repeating sugar unit in the O-specific side chains of smooth LPS. Types A and B differed only in the length of the laddered appearance. Isolates of type A, such as isolate 81 (refer to Figure 4.1, lane 1), had a small number of bands in the laddered appearance. This laddering was caused by the presence of a small number of repeating sugar units in the O-specific side chain. Isolates of type B, such as isolates 46, 54, 68, 76, 87 and 98 (refer to Figure 4.1, lanes 3-8 inclusive) showed the more common wild type LPS with a large number of bands representing the presence of a larger number of repeating sugar units in the O-side chain (refer to Figure 4.1, type B).

Type C represented by isolate 93 (refer to Figure 4.1, lane 2) demonstrated a distinct laddered appearance in which some of the bands were observed as doublets with the lower band of the doublet more intense or bands observed as triplets, with the middle band the most intense.

In addition, the type C LPS had a difference in the mobility of the core oligosaccharide band, which indicated it was a different size compared with the core oligosaccharides of type A and B isolates. The isolates with type C LPS had a core oligosaccharide band that migrated less (slower) through the SDS-polyacrylamide gels and therefore was larger in size.

Each sample was tested on at least two separate occasions to ensure the LPS type could be identified. However, there were some differences observed in the quality of the silver staining on different occasions. This resulted in some

discrepancies in the number of bands of the repeating-sugar units that were visualized.

Figure 4.1 is a polyacrylamide gel showing the LPS types of *S. virchow*. Table 4.2 lists each of the *S. virchow* isolates and the type of LPS observed.

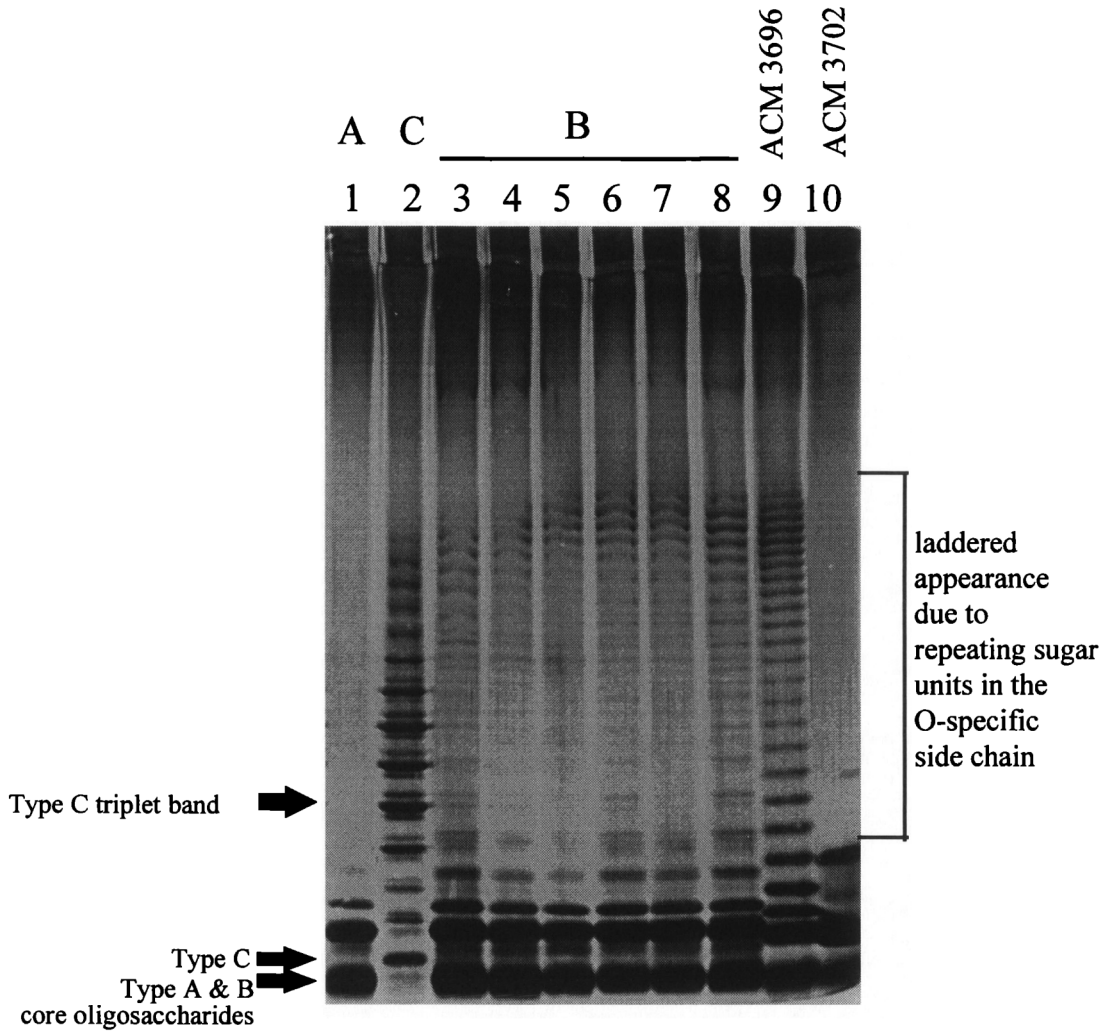


Figure 4.1 - Silver stained polyacrylamide gel showing LPS types. Lane 1 - isol. 81 (type A), lane 2 - isol. 93 (type C), lane 3 - isol. 46 (type B), lane 4 - isol. 54 (type B), lane 5 - isol. 68 (type B), lane 6 - isol. 76 (type B), lane 7 - isol. 87 (type B), lane 8 - isol. 98 (type B), lane 9 - *S. enteritidis* ACM 3696 (smooth LPS), lane 10 - *S. enteritidis* ACM 3702 (rough LPS)

Table 4.2 - Results of the LPS analysis of *S. virchow* isolates

Isolates with Type A Short - Medium length O - side chain	**Isolates with Type B Long O - side chain**	**Isolates with Type C Altered core region and doublets/triplets**
39, 40, 41, 42, 43, 44, 45, 58, 60, 61, 63, 64, 67, 78, 79, 80, 81, 83, 84, 85	1, 2, 3, 4, 5, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 29, 30, 31, 33, 34, 35, 36, 37, 38, 46, 47, 50, 51, 52, 53, 54, 55, 68, 69, 71, 72, 73, 74, 76, 77, 87, 88, 89, 91, 92, 94, 95, 96, 97, 98, 99, 101	8, 21, 28, 48, 56, 57, 59, 62, 66, 75, 82, 86, 90, 93, 100
20/95 = 21%	**60/95 = 63%**	
80/95 = 84%		**15/95 = 16%**

4.3. b) Investigation of variation in LPS structure

The following results were obtained from experiments to identify the cause of the changes to the LPS patterns observed, when new samples were prepared.

The investigation of the effect of subculturing, using freeze-dried, glycerol stocks and slope cultures of the isolates, showed that all samples regardless of subculture, had wild type, long chain LPS (type B). Figure 4.2 is a polyacrylamide gel showing several of the isolates from the various subcultures.

Experiments were performed to investigate if incubation time of the cells alters the LPS structure. Isolates were grown at the same temperature, for either 24 or 48 hours. No difference in the LPS type was found and all isolates regardless of incubation time showed long chain laddered LPS.

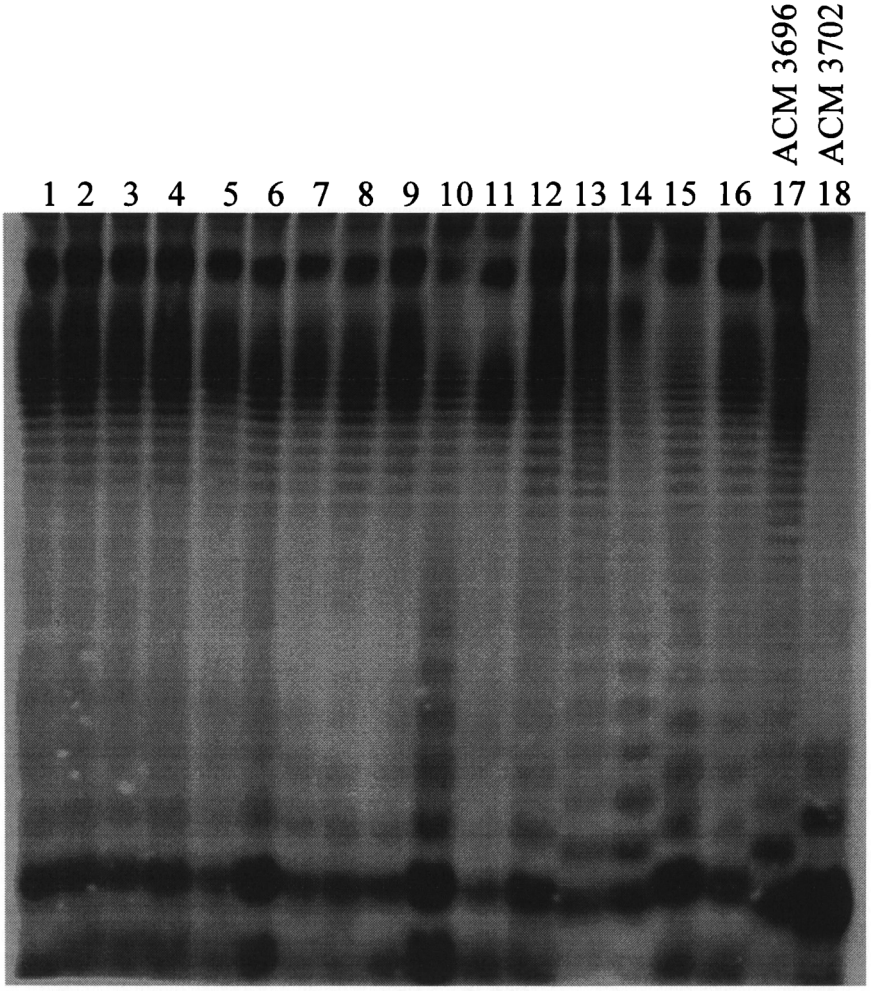


Figure 4.2 - Polyacrylamide gel showing the LPS structure of samples prepared from the freeze-dried, glycerol-stored and slope cultures. Lane 1 - isol. 2 : freeze-dried, lane 2 - isol. 2 : glycerol, lane 3 - isol. 2 : slope culture, lane 4 - isol. 7 : freeze-dried, lane 5 - isol. 7 : glycerol, lane 6 - isol. 7 : slope culture, lane 7 - isol. 39 : freeze-dried, lane 8 - isol. 39 : glycerol, lane 9 - isol. 39 : slope culture, lane 10 - isol. 44 : freeze-dried, lane 11 - isol. 44 : slope culture, lane 12 - isol. 8 : freeze-dried, lane 13 - isol. 33 : glycerol, lane 14 - isol. 33 : slope culture, lane 15 - isol. 38 : freeze-dried, lane 16 - isol. 38 : slope culture, lane 17 - *S. enteritidis* ACM 3696 (phage type 4), lane 18 - *S. enteritidis* ACM 3702 (phage type 7).

4.4 Discussion

The role of wild type LPS as a virulence factor is well documented (Chart *et al.*, 1989, 1991b; Finlay & Falkow, 1989; Guard-Petter *et al.*, 1995). Wild type LPS, also called smooth LPS, has an O-specific chain attached to the lipid A-core oligosaccharide complex. The chemical structure of the O-side chain consists of repeating sugar units and the number and sequence of sugars in the chain is unique and characteristic for *Salmonella* serotypes (Morrison & Ryan, 1992). The presence of these repeating units is what gives this LPS molecule its laddered appearance after polyacrylamide gel electrophoresis and silver staining. Rough LPS is the name given to LPS molecules which lack the O-specific chain (Morrison & Ryan, 1992). These molecules do not have the laddered appearance on polyacrylamide gels. The lack of an O-side chain has also been shown to correlate with avirulence of strains. Chart *et al.* (1991b) showed that strains belonging to phage types 7, 23 and 30 of *S. enteritidis* which had rough LPS were avirulent for BALB/c mice. Therefore the objective was to determine the LPS structure of the *S. virchow* isolates and establish if differences in the LPS correlated with the source information of the isolates.

Ninety five isolates of *S. virchow* were examined for their LPS structure and all were shown to produce wild type, laddered, smooth LPS. This indicated that all of the isolates were potentially virulent. However, three different smooth LPS types were identified by subtle differences in the laddered appearance. The most common type was type B (63%), which was the true wild type LPS which appeared the same as the control smooth strain, *S. enteritidis* ACM 3696. The second most common type was type A (21%) which differed from type B only in the number of bands visualized in the O-specific side chain. Members of type A had a short laddered appearance due to a small number of repeating sugar units in the O-side chain.

Heterogeneity in the number of bands representing the O antigen has been reported previously for *S. typhimurium* (Palva & Mäkelä, 1980). In that study, two thirds of *S. typhimurium* strains showed rough LPS with no side chain. The remaining third had smooth LPS and variation was observed with respect to the O-side chain lengths. The number of bands detected ranged from 19 - 34. It has been

suggested that the reason for this heterogeneity is that the translocase enzyme, which is encoded within the *rfb* gene cluster (Xiang *et al.*, 1993), is not specific in its activity (McConnell & Wright, 1979; Palva & Mäkelä, 1980). The function of the translocase is to transfer the polymerized O-units from a lipid carrier (undecaprenyl phosphate) to the core-lipid A. A lack of specificity in its activity results in different organisms synthesizing LPS with different O-side chain lengths. This was observed in the current study with different isolates of *S. virchow* being assigned to type A or B based on different O-side chain lengths.

Growth temperature has also been suggested as a cause of heterogeneity in the O-side chain. Investigations into the LPS structure of *Salmonella anatum* found that LPS from cells grown at 20°C had many more R-core units without O-side chains than LPS from cells grown at 30-35°C (McConnell & Wright, 1979). This was determined by differences in the sensitivity of the different cells to the rough-specific bacteriophage Felix 0-1.

Kawahara *et al.* (1989) showed that plasmid-encoded factors may also affect the side-chain. A regulatory factor which altered the chain length of the O-side chain was found to be encoded on the high molecular weight serotype-specific virulence plasmid of a *S. dublin* strain. The plasmid-cured strain had semi-rough LPS but when the virulence plasmid was reintroduced, the strain produced smooth type LPS. This was only seen in one strain of *S. dublin*, while other strains of this serovar and strains of *S. enteritidis*, *S. typhimurium* and *S. cholerasuis* did not show changes in LPS structure after curing and reintroduction of their virulence plasmids. Therefore, it was concluded that a regulatory factor and not a structural component, was encoded on the virulence plasmid. The *S. virchow* isolates in this study have not been thoroughly investigated for the presence of a high molecular weight virulence plasmid which may encode such a regulatory factor. Preliminary work indicated that a very small number, if any, may contain a high molecular weight plasmid (results not shown). Correlation between the possession of low molecular weight plasmid DNA and LPS type was only observed for one plasmid profile. The three members of PP2, isolates 43, 44 and 45, all produced type A LPS. However type A LPS was also produced by isolates from PPs 1, 3, and 6. Therefore no exclusive link between the presence of PP2 plasmid DNA and type A LPS could be concluded.

The third LPS type identified (type C) also had wild type LPS but was distinguished from types A and B by the presence of a larger core oligosaccharide band. Hitchcock and Brown (1983) demonstrated that electrophoretic migration patterns accurately reflect the biochemical differences of LPS molecules. Therefore, identification of strains with different relative mobility of the fastest migrating band, represent strains with altered core oligosaccharide. Core-defective mutants with shortened core oligosaccharide were shown by Hitchcock and Brown (1983) to have increased mobility. Therefore, the observation that the fastest migrating band of the type C isolates had decreased mobility indicates that these isolates had a larger core oligosaccharide. The other distinctive feature of the type C LPS was the appearance of some of the bands of the O-side chain as doublets and triplets.

Chart & Rowe (1995) were the first to describe intra-strain heterogeneity of LPS and this was demonstrated in *S. virchow*. A single strain was shown to express two different patterns, 50% of colonies showed doublet rungs while the other 50% had singlet rungs in the ladder. Both of these LPS types reacted to serum containing antibodies to purified *S. virchow* LPS and also to O=7 specific antigen. Chart & Rowe (1995) proposed that the difference within a strain indicated that *S. virchow* may have two distinct pathways of biosynthesis and assembly of LPS chains. This hypothesis could be supported by the conclusions of Lee *et al.* (1992) and Xiang *et al.* (1993) which indicated that the members of serogroup C_1, which includes *S. virchow,* have *rfb* genes which show little homology to the *rfb* genes of other serogroups. In particular, the two genes from the *rfb* cluster which encode the mannose pathway in the C_1 group are not closely related to the mannose pathway genes of groups A, B, D and E_1. However it is acknowledged that there are many other O antigen groups and it may be that the *rfb* region of C_1 has homology with one of the groups not yet studied.

It is proposed that the isolates with type C LPS in this study represent strains which Chart & Rowe (1995) described. The doublet and triplet appearance may be the result of the LPS sample being prepared using a mixed culture of cells. The LPS samples were prepared by harvesting cells that were patch inoculated from a slope culture and it is possible that the patch consisted of a mixed population, some with doublet appearance and some with singlet. The result of the two different types

being present in one sample would be the two patterns superimposed over each other when the sample was loaded on a polyacrylamide gel. In addition, the presence of more than one biosynthetic pathway may also explain the correlation between the presence of doublets and triplets and the larger core oligosaccharide. Perhaps the altered core is also a result of the synthesis or assembly of LPS by a different pathway.

The type C LPS requires further investigation to confirm the hypothesis that a mixed culture of cells was responsible for the appearance of the type. The first step would be to use single colonies of strains to prepare LPS samples for analysis, instead of patch inoculated growth. However even this approach may not provide a definitive answer. If single cells within the colonies have the potential to switch between pathways then even a single colony could result in a mixed LPS pattern.

Technically, the most difficult aspect of the LPS analysis was maintaining the quality of the silver staining. In particular, difficulties arose if an isolate was designated type B and on repeated testing poor silver staining resulted in less bands of the O-side chain being visualized. Under these circumstances an isolate would appear to be type B on one occasion and type A on another. The final judgement of the LPS type was made after consideration of the overall quality of the staining on each gel. In contrast the visualization of the type C LPS structure was less affected by the quality of the silver staining, probably because the distinctive features (doublets and altered core oligosaccharide bands) were more easily discernible.

The silver staining method used in the present study was that of Fomsgaard *et al.* (1990) and this method is a modification of the method of Tsai and Frasch (1982). Kittelberger and Hilbink (1993) reported another method of silver staining LPS in polyacrylamide gels which utilised a protein staining method after the periodate oxidation of the lipopolysaccharides. This was trialed in the present study but was more laborious and did not result in improved staining or resolution. Guard-Petter *et al.* (1995) reported a second variation on the method of Tsai and Frasch (1982) which was slightly different to the method used in the present study. This second modification used the same oxidation and staining but the development was done with a commercial kit, following the manufacturer's instructions. The difference between the commercial developer and that used by both Tsai and Frasch (1982) and

Fomsgaard *et al.* (1990) was that the kit uses paraformaldehyde instead of formaldehyde. Bands that had previously not been visualized on gels were detected when the same gels were restained with the commercial developer. It is likely that the use of this method would have been beneficial in the present study and this should be considered for future studies.

A further difficulty arose when samples of some of the isolates were prepared from the slope cultures on a second occasion. Analysis of the second set of LPS preparations showed that the LPS pattern of all isolates previously recorded as types A and C were now observed as type B. All the original type B isolates maintained their LPS pattern. The significance of the change for types A and C could not be determined because these types had not been reported in the literature previously. Since types A, B and C were all smooth type it was not obvious whether the subtle differences that distinguished them effect the virulence of these strains. To further investigate the change of LPS type that was observed, a subgroup of isolates was chosen and several hypotheses tested.

The effect of incubation time on LPS type was investigated in preliminary experiments. Isolates were grown for either 24 or 48 hours on TSA plates before LPS samples were prepared. The results showed that regardless of the incubation time all isolates had type B LPS. However, it is likely that the 24 hour difference in incubation time used in this study was not sufficient to observe a change in the LPS type. This should be repeated using larger variations of incubation times such as 72 hours, 96 hours and up to several weeks.

Temperature of incubation has been shown to effect the expression of LPS with respect to the length of the side chain (McConnell & Wright, 1979). The approach taken in this study was to eliminate temperature as a variable by keeping incubation temperature constant between samples. However, the alternative approach would be to perform the incubation of cultures at different temperatures before preparing the samples and directly comparing the LPS types of the same isolate when grown at different temperatures.

It was also proposed that subculture in the laboratory caused changes in the expression of LPS. To investigate this hypothesis cultures from different stages of subculture were tested. The material tested included freeze-dried preparations which

had been stored at 4°C and glycerol stocks which had been stored at -70°C. The glycerol and freeze-dried cultures had both undergone a lesser number of subcultures before storage than the slope cultures that had been used to prepare the initial LPS samples. Results of these experiments showed that all LPS samples had wild-type long-chain LPS (type B). These findings indicated that laboratory subculturing alone did not explain the changes observed. Nevertheless, the hypothesis that laboratory subculturing does cause change of the smooth LPS pattern should be investigated by a time course study. Isolates expressing wild type LPS (type B) should be subcultured routinely over a long period and LPS samples prepared at each subculture to determine if any changes in the LPS type occur. This should also be done as described earlier for testing of type C isolates by testing individual colonies rather than patch inoculated growth.

Further, the hypothesis that smooth LPS patterns are effected by the culturing environment of the bacteria should be tested by passaging isolates through cell culture lines and/or mice. The rationale of these experiments is to mimic the *in vivo* interactions between *S. virchow* and host cells to observe whether LPS expression is altered.

This investigation of the LPS structure of *S. virchow* isolates has demonstrated heterogeneity with respect to the O-side chain length, similar to that previously reported for other *Salmonella* strains. In addition, some isolates demonstrated an LPS pattern (type C) that indicates there may be more than one pathway of LPS biosynthesis and assembly, as reported by Chart *et al.* (1995). Isolates were also observed to express different LPS patterns when tested at different times and a hypothesis was suggested that expression of LPS is altered for cells *in vitro*.

Future studies should first confirm the type C LPS pattern by testing single colonies to observe the two different patterns that combine to generate the doublets and triplets. Also a time course study should be undertaken to elucidate how the expression of LPS in these strains is effected by their environment.

In addition, investigation of the pathways involved in LPS biosynthesis in *S. virchow* and other members of the C_1 serogroup would be important as it seems

likely they behave differently to other serogroups. Perhaps the first objective should be to determine the LPS patterns of other C_1 serogroup members, such as *S. infantis* and determine if intra-strain heterogeneity exists in these strains. The control mechanism for the biosynthetic pathways would also be an important finding. To determine what factors control the pathway of LPS biosynthesis and assembly in different cells of the same strain or at different times and in different environments, would potentially provide great insight into the relationship between LPS structure and the virulence of these strains.

CHAPTER 5

CELL CULTURE INVASION ASSAY

Chapter 5 : Cell Culture Invasion Assay

5.1 Introduction

Salmonella serovars are known to be associated with different types of disease in humans, ranging from typhoid fever caused by *S. typhi*, to gastroenteritis. *S. virchow*, has been associated with both diarrhoeal and extraintestinal infections, such as septicaemia and osteomyelitis (Mani *et al.*, 1974; Todd & Murdoch, 1983; Taha & Peden, 1987; Ingram & Redding, 1988; Ashdown & Ryan, 1990; Sechter *et al.*, 1991) The transmission and progression of infection is by the same route for all of these different disease states. This involves ingestion of the organism followed by invasion of the gut epithelium. The ability to adhere to and enter cells, both the intestinal epithelial cells and macrophages, is central to the pathogenesis of *Salmonella* infection. Hence cell culture assays have become valuable *in vitro* tools to study invasiveness of strains. Many serovars have been studied using cell culture including *S. typhimurium*, *S. cholerasuis* and *S. derby* (Tavendale *et al.*, 1983; Finlay & Falkow, 1988; Finlay *et al.*, 1989; Gahring *et al.*, 1990; Budiarti *et al.*, 1991; Douce *et al.*, 1991). Galán and Curtiss III (1991) are the only researchers to report testing *S. virchow* using cell culture and that was only one strain in the Henle-407 cell assay.

Douce *et al.* (1991) used a simple cell culture invasion assay to study the invasiveness of *S. typhimurium* and the objective of the current study was to compare the behaviour of *S. virchow* using the same assay. It was hoped that *S. virchow* isolates would show significant differences in their ability to invade by this assay.

Previous chapters of this study have examined two putative virulence factors of *S. virchow*, SEF 17 fimbriae and LPS. These factors may be involved in the invasive infection associated with this serovar. A cell culture assay which could quantitate invasion would provide a valuable method to measure changes in the invasive ability of strains that have undergone genetic manipulation of virulence factors. In addition, a cell culture assay would also be preferable, in terms of ethical considerations, to the alternative methods for comparison of virulence; mouse models and the rabbit ileal loop test.

5.2 Materials and Methods

5.2. a) Maintenance of the HEp-2 cell line

5.2. a (i) Cell line

The HEp-2 cell line (human larynx epithelium) was used to perform the invasion assays and was chosen primarily because it had been in other studies, the majority of which were studying *S. typhimurium* (Tavendale *et al.*, 1983; Budiarti *et al.*, 1991; Douce *et al.*, 1991; Jones, B. D. *et al.*, 1992; MacBeth & Lee, 1993; Bäumler *et al.*, 1996). HEp-2 is an epithelial cell line making it appropriate as a model of the cell type that *Salmonella* would invade. The seed was provided by Dr P. R. Young (Sir Albert Sakzewski Virus Research Centre).

5.2. a (ii) Media and solutions

The media used was Dulbecco's Modification of Eagle's Medium (DMEM) which was purchased as a dehydrated powder (Catalogue No. 10-331-20, ICN Biomedicals). All components were filter sterilized, except the foetal calf serum which was heat inactivated and added just prior to use. Antibiotics were included in the media for routine passaging but omitted from media used in invasion assays.

Components of cell culture media :

DMEM powder (ICN Biomedicals)	1 sachet
Sodium Hydrogen Carbonate	3.7g
Hepes (1M)	20ml
Tissue Culture grade water	877ml
Foetal calf serum (heat inactivated)	100ml
L-glutamine (200mM)	2ml
Streptomycin/Penicillin (50U/50μg/ml respectively)	1ml
	1000ml

Dulbecco's modification of phosphate buffered saline (DPBS)

Sodium chloride	8g
Potassium chloride	0.2g
Disodium hydrogen orthophosphate	1.15g
Potassium dihydrogen phosphate	0.2g
Tissue culture grade water	1000ml

pH 7.2 and sterilized by autoclaving 121°C for 20 minutes

Prepared Trypsin-EDTA solution (0.05% (w/v) trypsin and 0.02% (w/v) EDTA in salt solution, Catalogue No. 16891-49, ICN Biomedicals) was used.

5.2. a (iii) Routine passaging of monolayers

Confluent monolayers in $25cm^3$ flasks were passaged and split routinely at 1:3 or 1:4 dilutions. Passaging was performed as follows :

1. Media including the DMEM, DPBS and Trypsin-EDTA were pre-warmed to 37°C
2. Spent media was removed from the flasks containing the confluent monolayer and the monolayer was rinsed by adding 10ml of DPBS
3. After gently rinsing the monolayer and the walls of the flask the DPBS was removed and 2ml of Trypsin-EDTA was added, used to rinse the monolayer and then discarded
4. A second 2ml of Trypsin-EDTA was added and the flask was incubated at 37°C/5% CO_2 for ca. 5 minutes
5. After 5 minutes, the monolayer was checked for lifting and rounding of the cells. Lifting was assisted by gently rocking the flask and tapping the sides. Trypsin-EDTA was not allowed to remain on cells for any longer than necessary
6. Once cells had lifted, 5.2ml of DMEM was added and the volume was pipetted up and down to thoroughly resuspend the cells

7. The cell suspension was then evenly distributed between a number of fresh flasks depending on the severity of the split and extra DMEM was added to each resulting flask to a total volume of 10ml per $25cm^3$ flask. This was also the correct cell density to inoculate a 24 well plate for the invasion assay by adding 300µl to each well and adding 700µl of DMEM to fill the wells
8. Flasks were incubated 37°C with 5% CO_2 with the lids loosened to allow gas exchange until either the media was exhausted (indicated by the colour change due to acid accumulation) or the monolayer again reached confluence.

5.2. b) Storage and Resuscitation of HEp-2 cells

5.2. b (i) Preparation of cryovials for liquid nitrogen storage

1. Cell monolayers were trypsinized as outlined above (refer to section 5.2a (ii)) but instead of dispensing the resultant cell suspension between new flasks, the suspension was added to a 10ml centrifuge tube and spun at 1200 rpm for 5 minutes to gently pellet cells
2. The supernatant was removed and the cells were resuspended in 3ml of DMEM and pipetted up and down to mix the cells
3. 100µl was removed and mixed with 300µl of Trypan blue dye and the viable cell concentration was determined using a haemocytometer chamber
4. Cell suspensions were either diluted or concentrated to get a cell concentration of 2.5-4 x 10^6 cells/ml
5. Suspensions of the desired concentration were cooled on ice along with the cryovials to be used
6. 0.9ml of cell suspension was pipetted into each cryovial and 0.1ml dimethlysulfoxide (DMSO) was added (cells must be cold at this stage because DMSO is toxic to cells at room temperature)

7. Working quickly, the cryovials were wrapped in cotton wool and placed at -20°C for one hour followed by -70°C for 3-4 hours or overnight
8. Finally the cryovials were placed in liquid nitrogen storage.

5.2. b (ii) Resuscitation of cells from liquid nitrogen storage

1. The cryovial was removed from liquid nitrogen and thawed almost completely at 37°C
2. Each 1ml of thawed cells was immediately added to 9ml of fresh DMEM in a centrifuge tube (this needs to be quick because the DMSO is toxic to the cells)
3. The centrifuge tube was spun at 1200rpm for 5 minutes or until a pellet of cells was visible
4. The supernatant was removed and the cells resuspended in 9-10ml of DMEM and added to a flask
5. The flask was incubated at 37°C with 5%CO_2
6. The media was changed after ca. 24 hours incubation. The monolayer was not split in the first few days, but was gently lifted off and allowed to resettle.

5.2. c) Invasion assay

5.2. c (i) Preparing the HEp-2 monolayer

The seeding of the HEp-2 cells into a 24-well plate was done 24 - 36 hours prior to an invasion assay being performed to allow a monolayer to develop. The procedure for seeding this monolayer is below :

1. Sterile glass coverslips were placed in appropriate wells, using forceps, prior to seeding so that monolayers would grow on the coverslips to allow microscopy after the invasion assay,

2. Cells from a confluent $25cm^3$ flask (refer to section 5.2a (ii)) produced one 24 well plate of seeded wells by adding 300µl aliquots of cells to each well using an automatic pipette to ensure reproducible volumes
3. An additional 700µl of DMEM, without penicillin/streptomycin, was added to each well and the plate was incubated at 37°C with 5% CO_2 for 24 - 36 hours, to allow the cells in each well to become confluent
4. Once the monolayers were formed (monolayers were not used once incubated for over 40 hours) a well was sacrificed and used to determine the cell density (refer to section 5.2b i).

5.2. c (ii) Preparing the bacterial inoculum

Growth of the bacterial isolates for an assay began 24 hours prior to the assay by patching the isolates onto a tryptone soy agar (TSA) plate and incubating at 37°C. After incubation the bacterial inoculum was prepared to an approximate concentration of 1×10^7 cell/ml as follows

1. A small loopful of growth was transferred from the overnight plate culture to 2ml of sterile TE buffer (Sambrook *et al.*, 1989) and vortexed
2. 100µl of the suspension was transferred into 15ml of sterile tryptone soy broth (TSB) and using sterile TSB as a blank, the A_{620} of the suspension was determined. A_{620} of between 0.001 - 0.01 was approximated 1×10^7 cells/ml. If the concentration was not high enough more of the cells from the TE suspension were added until the A_{620} was within the desired range
3. Dilutions of this bacterial inoculum were prepared (10^{-4}, 10^{-5} and 10^{-6}) and plated in duplicate onto TSA and incubated overnight. On the following day colony counts were performed on these plates to determine the exact bacterial inoculum used.

5.2. c (iii) Assay Protocol

The assay protocol was developed after many experiments to determine the effect of the following variables :

- the size of the bacterial inoculum (bacterium:cell ratio),
- concentration and contact time for gentamicin sufficient to kill extracellular bacteria,
- length of contact time for bacterial cells with the HEp-2 cells; and
- reproducibility.

These factors and their optimization are described in the discussion (refer to section 5.4) and the following is the final protocol :

1. 1ml of the prepared bacterial suspension (10^7cfu/ml) was added to wells containing confluent HEp-2 monolayers (each strain was added to 3 wells, 2 without a coverslip and 1 with a coverslip) and incubated for 3 hours at 37°C with 5% CO_2
2. After 3 hours, the bacterial suspension was removed and 1ml of pre-warmed (to 37°C) DPBS was added and pipetted up and down to rinse cells, then discarded
3. 30µl of gentamicin (10mg/ml) and 300µl of pre-warmed DMEM was added to each well and incubated at 37°C/5%CO_2 for 30 minutes
4. The gentamicin/DMEM was then removed and the wells were again washed with 1ml of pre-warmed DPBS (this was very important to wash away any dead extracellular cells from the coverslips),
5. Using a flamed wire, from the underside of the plate, a hole was pierced in the wells that contained coverslips to lift them from the bottom and the coverslips were then removed using forceps and put aside to be stained later
6. 1ml of Trypsin-EDTA added to the remaining wells and allowed to sit for several minutes
7. The monolayer was then agitated by pipetting up and down with an automatic pipette to ensure all of the cells were dislodged and the

suspension was transferred to a sterile 10ml centrifuge tube. The tube was centrifuged at 2000 rpm for 5 mins

8. The supernatant was removed and the cell pellet was resuspended in 1ml of DMEM and 200µl of Triton-X 100 (1%). The tube was vortexed briefly and then allowed to stand at room temperature for 5 minutes
9. Dilutions of the suspension were prepared in sterile saline and spread plated onto TSA. The plates were incubated at 37°C overnight and colony counts were performed the following day to determine the invasion rate
10. The coverslips that were set aside from the wells were stained using the Diff-Quik staining kit (Lab-Aids Pty Ltd, Australia), following the manufacturer's instructions. The coverslips were permanently mounted onto glass microscope slides using DePex mounting medium (BDH) and were viewed using x100 oil immersion light microscopy.

5.2. c (iv) Calculation of results

The day after the assay, the colonies were counted on the spread plates from the Triton-X treated HEp-2 cells to calculate the number of bacterial cells which had invaded the HEp-2 cells. The dilution plates of the bacterial inoculum were also counted to determine the number of bacteria added to the monolayer. From these counts the percentage invasion for each strain was calculated using the following equation, as proposed by Douce *et al.* (1991) :

$$\text{percentage invasion} = \frac{\text{no. bacterial cells isolated from HEp-2 cell}}{\text{no. bacteria inoculated onto HEp-2 cells}} \times 100$$

5.2. c (v) Isolates tested and statistical analysis

The only basis on which the *S. virchow* isolates could be judged for their potential invasiveness was by the source data available. Therefore isolates were identified as invasive or non-invasive based on their site of isolation. The rational being that an isolate which was isolated from blood must have been capable of invasion

of the reticuloendothelial system. In contrast, faecal isolates were considered non-invasive. Table 5.1 shows the isolates tested using the invasion assay.

The results of the invasion assay were recorded as the percentage invasion and statistical analysis using the SAS system was performed by Dr Joan Hendrikz, Stats Advisor, University of Queensland. Log_{10} of each of the percentage invasion values were used in the analysis but because the values were so low the resulting log_{10} values were negative. To overcome this, a constant of 3 was added to all results. This was the same as multiplying the percentage invasion by 10^3 before taking the log. It altered the means and standard deviations calculated but it made no difference to the outcome because it was a constant factor.

Table 5.1 - Isolates used to evaluate the HEp-2 cell culture invasion assay

Isolate	**Site of isolation**	**Invasive/Non-Invasive**
2	frog's legs	Invasive
8	chicken meat	Invasive
16	chicken meat	Invasive
57	human urine	Invasive
63	wallaby liver	Invasive
68	porcine pus	Invasive
92	human blood	Invasive
107	human urine	Invasive
109	human chest wall	Invasive
1	meat/bone meal	Non-Invasive
3	possum	Non-Invasive
4	human faeces	Non-Invasive
5	macadamia nuts	Non-Invasive
33	chicken faeces	Non-Invasive
35	human faeces	Non-Invasive
48	chicken faeces	Non-Invasive
55	human faeces	Non-Invasive
83	human faeces	Non-Invasive
102	human faeces	Non-Invasive
120	chicken faeces	Non-Invasive
S. typhimurium 82/6915	poultry Vic	Invasive

Two-way main effects analysis of variance (ANOVA) was done to take into account the inter-assay variation for each isolate and the inter-isolate variation. The

critical value to determine significance was $P<0.05$. The isolates were then grouped into, invasive and non-invasive groups. A three factor ANOVA was performed, taking into account the inter-assay variation, to determine if there was any significant difference in the rate of invasion between the two groups.

S. typhimurium 82/6915 was included in each assay as a positive control because it had been shown to be invasive in a HeLa cell culture assay developed at RMIT (Sarah-Jane Rickard - pers. comm.). A t-test was used to determine if any significant difference existed between the rate of invasion of *S. typhimurium* compared to the invasive and non-invasive groups of *S. virchow*. The t-test was for two samples assuming unequal variances, at the 95% confidence level. This statistical comparison was performed using Microsoft Excel Version 5.0c.

5.3 Results

5.3. a) Invasion assay

A total of nine invasive isolates and eleven non-invasive isolates were tested using the cell culture assay. Table 5.2 shows the results for the invasive group and Table 5.3 shows the results for the non-invasive isolates. The results represent each assay that was performed showing the percentage invasion in each well and the average percentage invasion by each isolate per assay.

Table 5.4 contains the results for *S. typhimurium* 82/6915 which was an invasive control strain included in each assay (known to be invasive in HeLa cell culture invasion assays (Pers. comm. - Sarah Rickard). No result was obtained for *S. typhimurium* 82/6915 in assay H.

Table 5.2 - Invasion assay results of the invasive isolates

		Percentage invasion (%)								
Assay		**2**	**8**	**16**	**57**	**63**	**68**	**92**	**107**	**109**
F	wells	0.065								
		0.059								
		0.043								
	Avg.	**0.056**								
G	wells	0.013								
		0.017								
		0.02								
	Avg.	**0.017**								
H	wells		0.025					0.525		
			0.017					0.179		
	Avg.		**0.021**					**0.352**		
I	wells		0.107					1.11		
			0.054					1.95		
	Avg.		**0.081**					**1.53**		
J	wells		0.15					0.758		
			0.15					1.22		
	Avg.		**0.15**					**0.989**		
K	wells				0.206			0.075	0.019	
								0.139	0.535	
	Avg.				**0.206**			**0.107**	**0.277**	
N	wells						0.314	0.352	0.408	
							0.266	0.505	0.398	
	Avg.						**0.290**	**0.429**	**0.403**	
P	wells		0.017							
			0.018							
	Avg.		**0.018**							
Q	wells		0.018			0.049			0.064	
									0.035	
	Avg.		**0.018**			**0.049**			**0.050**	
R	wells		0.16	0.025	0.023				0.113	0.032
			0.19	0.021	0.036					
	Avg.		**0.18**	**0.023**	**0.030**				**0.113**	**0.032**
S	wells			0.019	0.008		0.007			0.074
					0.013		0.014			0.068
	Avg.			**0.019**	**0.011**		**0.011**			**0.071**
Average for each isolate		**0.036**	**0.077**	**0.021**	**0.082**	**0.049**	**0.150**	**0.681**	**0.211**	**0.052**
Average of all isolates		**0.151**								

Table 5.3 - Invasion assay results of the non-invasive isolates

		Percentage invasion (%)										
Assay		**1**	**3**	**4**	**5**	**33**	**35**	**48**	**55**	**83**	**102**	**120**
F	wells			0.091								
				0.113								
				0.075								
	Avg.			**0.093**								
G	wells	0.022	0.017	0.046								
		0.017	0.016									
		0.021										
	Avg.	**0.02**	**0.017**	**0.046**								
H	wells							0.067				
								0.096				
	Avg.							**0.082**				
I	wells	0.107	0.18		0.01		0.361	0.166				
		0.117			0.015		0.406	0.244				
	Avg.	**0.112**	**0.18**		**0.013**		**0.384**	**0.205**				
J	wells	0.151			0.003		0.19	0.162				
		0.044			0.008			0.134				
	Avg.	**0.098**			**0.006**		**0.19**	**0.148**				
K	wells					0.064		0.016			0.065	0.016
						0.071		0.016			0.074	
	Avg.					**0.068**		**0.016**			**0.07**	**0.016**
N	wells							0.362			0.391	
								0.353			0.516	
	Avg.							**0.358**			**0.454**	
P	wells	0.006										
		0.005										
	Avg.	**0.006**										
Q	wells								0.027	0.024	0.039	0.022
									0.038	0.041	0.059	0.028
	Avg.								**0.033**	**0.033**	**0.049**	**0.025**
R	wells					0.005			0.02	0.67	0.598	1.74
									0.03	0.085	2.28	0.223
	Avg.					**0.005**			**0.025**	**0.378**	**1.44**	**0.982**
S	wells		0.079		0.002		0.027		0.051	0.007		
			0.005				0.022		0.053	0.005		
	Avg.		**0.042**		**0.002**		**0.025**		**0.052**	**0.006**		
Average for each isolate		**0.059**	**0.08**	**0.07**	**0.007**	**0.036**	**0.199**	**0.162**	**0.037**	**0.139**	**0.503**	**0.341**
Average of all isolates		**0.148**										

Table 5.4 - Invasion assay results for *S. typhimurium* 82/6915

Assay		*S. typhimurium* 82/6915
F	wells	2.45
		2.72
		1.47
	Avg.	**2.21**
G	wells	0.39
		0.29
		0.42
	Avg.	**0.37**
H	wells	
	Avg.	
I	wells	1.05
	Avg.	**1.05**
J	wells	2.43
		1.77
	Avg.	**2.10**

Assay		*S. typhimurium* 82/6915
K	wells	0.13
		0.015
	Avg.	**0.07**
N	wells	0.28
		0.22
	Avg.	**0.25**
P	wells	0.08
		0.06
	Avg.	**0.07**
Q	wells	0.06
		0.09
	Avg.	**0.08**
R	wells	0.008
		0.568
	Avg.	**0.29**
S	wells	0.279
		0.151
	Avg.	**0.22**
Average		**0.67**

5.3. b) Statistical Analysis

The results in Table 5.5 of the two-way main effects ANOVA clearly show that there was no significant variation between assays or between isolates. The critical value used was 0.05 and therefore a $P>0.05$ indicates no significance.

Table 5.5 - 2-way main effects ANOVA analysis results

Group	Source of variation	F value	P value
Invasive	inter-isolate	0.76	0.6337
	inter-assay	1.35	0.3416
Non-invasive	inter-isolate	1.98	0.1237
	inter-assay	1.66	0.1944

The 3 factor ANOVA and an F test were performed to compare the percent invasion for the isolates when the results where not pooled together by invasive and non-invasive. The result of this analysis are presented in Table 5.6 and show that there was significant variation ($P<0.05$) between assays for individual isolates, which

was observed from the raw data in Tables 5.2 and 5.3. However, the variation was not significant for invasive versus non-invasive because the variation was the same for both groups. The F test showed that there was no significant variation in the percent invasion by the two pooled groups. These results are likely a reflection of the composition of the invasive and non-invasive groups. In real terms the allocations of isolates to these groups was arbitrary because isolates from faeces may have the potential to be equally as virulent as isolates from blood.

Table 5.6 - 3 factor ANOVA and F test results

Test	Source of variation	F value	P value
3 factor ANOVA	between assays	2.45	**0.0403**
3 factor ANOVA	between groups	0.25	0.9805
3 factor ANOVA	between isolates	1.52	0.1784
F test	between isolates % invasion	2.60	0.1379

A t-test was used to compare each of the pooled *S. virchow* groups to the pooled result for control strain, *S. typhimurium.* The results in Table 5.7 show that there was a significant difference ($P<0.05$) between the *S. typhimurium* and both the invasive group and the non-invasive *S. virchow* group. This highlights the fact that the percent invasion by the *S. typhimurium* strain was significantly higher than that of either of the *S. virchow* groups.

Table 5.7 - T-test results for *S. typhimurium* and *S. virchow*

Test	Source of variation	P value
t-test	between *S. typhimurium* and invasive *S. virchow*	**0.0442**
t-test	between *S. typhimurium* and non-invasive *S. virchow*	**0.0397**

5.4 Discussion

In the present study *S. virchow* isolates were tested for their level of invasion using a HEp-2 cell culture invasion assay. This assay method had been employed previously by others to study the invasiveness of *S. typhimurium* strains (Douce *et al.*, 1991). To allow comparison of *S. virchow* isolates to a known invasive isolate, a *S. typhimurium* strain that had been shown to be invasive by a similar assay in HeLa cells was included.

Gentamicin treatment during the assay was performed to kill extracellular bacterial cells. Optimization of the dosage and exposure time with gentamicin was performed using a matrix approach and testing 300, 500, 750 and 1000μg gentamicin per well and incubation times of 30 minutes, and hourly up to 5 hours. The final assay protocol used a fresh supply of gentamicin at a concentration of 300μg per well, incubated for 30 minutes.

Longer incubations (>1 hour) with gentamicin were performed to confirm whether intracellular replication of *S. virchow* was occurring. The ability of the *S. virchow* isolates to multiply within vacuoles of epithelial cells has implications for the performance of the invasion assay. The incubation period of the bacteria with the monolayer for the invasion assay, was routinely kept to 3 hours because if bacterial cells were in contact with the monolayer for too long they could enter the HEp-2 cells and if given sufficient time begin intracellular multiplication. In this situation the bacterial count obtained after gentamicin treatment and lysis of the HEp-2 cells would not be a true representation of the number of bacterial cells which had invaded the monolayer. It would also represent bacterial cells which had multiplied in the HEp-2 cells after invasion. A lag period of about 4 hours between entry of *Salmonella* into host cells and commencement of replication in the vacuoles of epithelial cells has been reported previously (Finlay *et al.*, 1992). The limited results for intracellular replication experiments performed in this study (data not shown) are in agreement with this observation as there was no significant intracellular replication in the first three hours after contact but after 5 hours slight increases in cell numbers were observed, indicating the beginning of replication.

In vivo, Salmonella replicate in the reticuloendothelial system of the host, not the epithelial cells (Finlay & Falkow, 1989). Therefore in the future it would be useful to test *S. virchow* isolates in an intracellular replication assay using macrophage cells lines instead of epithelial cell lines.

Another factor that was influential in the performance of the invasion assay was the age and confluency of the monolayer. It was important to ensure that the monolayer was at the same level of confluence on each occasion so that approximately the same number of HEp-2 cells were present. This was done by standardizing the volumes used in seeding the wells and incubating the plates until the monolayer was confluent but not hyperconfluent. It was not sufficient to incubate the wells for a set period because as the cells were passaged, their growth rate decreased and it took longer for the cells to reach confluence. In particular, once the cells reached approximately passage 30, a fresh aliquot from liquid nitrogen storage was retrieved.

An observations of Douce *et al.* (1991) was that when the bacterium:cell ratio of 100:1 and a 2 hour interaction time was used the percentage invasion was significantly less than 1%. This was also observed in the present study, in which the bacteria were inoculated at ca. 1 x 10^7 cfu/ml and the monolayer was routinely ca. 1-5 x 10^4 cells/ml at confluence, giving an even higher ratio of 1000:1. Despite the interaction proceeding for 3 hours, Tables 5.2 and 5.3 showed that the percentage invasion was often much lower than 1% of the inoculum for both the invasive and the non-invasive isolates of *S. virchow*.

A difficulty encountered by Douce *et al.* (1991) when a high bacterial inoculum was used was that a high concentration of bacteria was seen on the monolayer, using microscopic examination of stained monolayers. Interactions of greater than 2 hours also resulted in uneven distribution of the bacteria on the monolayer, many cells having no bacteria associated with them and others which had microcolonies of bacteria. This was also observed in the present study, in which the monolayers, stained with Diff-Quik, often showed large numbers of bacteria present in clusters on some cells and large areas of the monolayer where no bacteria were seen (data not shown). Douce *et al.* (1991) concluded that overall a lower inoculum was better, both because of the reduction in this effect and the more favourable ratio

of bacteria to cells. This use of a lower inoculum was tried in the present study but 10^5 cfu/ml did not result in any detectable invasion.

Douce *et al.* (1991) found that overnight cultures of bacteria were less invasive than cultures in log-phase. In this study both methods of preparing inoculum were tested and the percentage invasion that resulted were approximately equal. However the invasion rates were low and this could explain why no significant difference between the types of inoculum was observed. This may require further investigation when the sensitivity of the assay has been improved to ascertain why younger cells in log-phase might be more invasive. In the present study the use of cells from an overnight culture, resuspended to a desired absorbance value was found to be a reproducible method to prepare an inoculum of 10^7 cfu/ml and this was adopted.

The most discouraging aspect of the results was the lack of reproducibility. Douce *et al.* (1991) reported a similar difficulty with reproducibility only in the pattern of comparative strain behaviour and not in the absolute invasion values. The *S. virchow* isolates in this study also showed this trend. The results in Tables 5.2 and 5.3 show that there were differences between the results of wells for the same isolate in the same assay, as well as differences for an isolate when it was tested in different assays. However there were overall trends such as isolate 92 which generally had the highest rate of invasion for the isolates in the invasive group and isolates 2 and 16 were usually the least invasive from this group.

There was no significant difference in the level of invasion of the two groups of *S. virchow* isolates ($P = 0.138$). While there was not a large number of isolates tested using the invasion assay it was hoped that a more definite distinction between the invasive and the non-invasive isolates would occur. The most likely explanation for this lack of differentiation by invasiveness was that the two groups of isolates used were ill-defined and did not accurately reflect invasive and non-invasive isolates. At the time of performing these invasion assays the only determinant that was known about the isolates, on which they could be judged, was their site of isolation. It is likely that this was not an accurate indication of their potential to cause an invasive infection. The invasive group included some isolates from extracellular sites of human infection and these isolates were amongst those that showed higher rates of invasion. Isolate 92

for example, which was the most invasive isolate, was from human blood. The next most invasive isolate of the invasive group was also from an extraintestinal site, isolate 107 from urine. However the isolates which were not as invasive were isolates such as 2 and 16 which were from frog's legs and chicken meat, respectively. This suggests that while these isolates were classified as invasive in this study (refer to Table 5.1) they may in fact have been present at these sites not because of their invasive nature but instead by cross contamination during food handling or processing.

In addition, several of the isolates from the non-invasive groups had invasion rates that were equivalent to members of the invasive group suggesting that they may have the same potential to cause invasive illness but had been isolated from sites that did not indicate this. In particular, isolate 102 from human faeces showed percentages of invasion similar to isolate 92 from human blood, but had been deemed non-invasive.

Therefore a more accurate evaluation of the cell culture assay could be performed if a more accurately defined group of isolates was tested. One method to obtain a more appropriately defined group of isolates would be to subject them to the rabbit ileal loop test (RILT). The RILT determines the virulence of isolates based on their ability to induce fluid secretion. Alternatively isolates characterized with respect to virulence factors such as SEF17 fimbriae or LPS (see chapters 3 and 4) could provide distinct groups which might be appropriate for evaluation of the invasion assay. Unfortunately this was not possible in the current study as that information was not available at the time of the cell culture experiments.

Using the current groupings of isolates there was, however, a significant difference between the invasion rates of the *S. virchow* isolates and the *S. typhimurium* 82/6915 control strain. The average percentage invasion of the *S. typhimurium* strain was 4.5 times higher than that of the average for the invasive and non-invasive *S. virchow* groups. Again, this is an indication that if truly virulent and avirulent *S. virchow* isolates were tested it is more likely that a significant difference between the two groups would be observed.

It should be noted that the levels of invasion measured in this work were extremely low and this makes it very difficult to compare strains. As the assay was performed in this work, not a great deal of confidence can be placed in the assignment of invasive potential based on the percentage invasion measured. Many of the

suggestions that have been cited in this discussion should be tested to improve this assay.

Another application of this assay that could be considered for the future is screening isolates which have undergone genetic manipulation of virulence determinants to observe changes in the invasive ability of altered strains. Genetic loci present on the *Salmonella* chromosome which contain genes required by the bacteria for entry into host cells and also invasion loci have recently been reported (Galán, 1996). Genes present in the loci include those which encode well known surface structures such as LPS and flagella and others which encode regulatory factors for expression of surface structures and regulation of protein secretion pathways. Phylogenetic studies of the invasion loci suggested that *Salmonella* acquired the loci as a block from some other microorganism (Galán *et al.*, 1992; Ginocchio *et al.*, 1992). A consequence of this is that the locus is unstable and deletions of the entire locus have been reported in *S. lichtfield* and *S. seftenberg* (Galán, 1996). The consequence of loss of this invasion locus is naturally occurring non-virulent strains. Therefore genetic analysis could be used to determine if deletions occur in *S. virchow* and the cell culture assay could be used to confirm if the presence or absence of the invasion locus correlates with differences in the invasiveness of isolates. This should be done not only using the epithelial cell lines but macrophage lines as well.

CHAPTER 6

GENERAL DISCUSSION

6.1 General Discussion

The two features of *S. virchow* that made it a compelling subject of study were its prevalence in Queensland and its propensity to cause invasive infection.

The work presented in this thesis does not directly address the issue of the prevalence of *S. virchow* in Queensland. What was achieved was the application of several typing methods to *S. virchow* and evaluation of their relative discriminatory power. These results served to show that the *S. virchow* of poultry is a reservoir for human infection. The results also provided a basis on which to choose effective typing methods to apply in an investigation of the prevalence of *S. virchow* in Queensland.

If a study of the prevalence of *S. virchow* in Queensland is desired it would require a large scale survey of *S. virchow*. Such an investigation should include attempts to isolate *S. virchow* from many and varied locations and sources in Queensland, in particular far north Queensland because of the high incidence of *S. virchow* in this area, as well as other regions of Australia and overseas. The survey should also include screening of introduced and native species, as well as food animals such as poultry and beef to identify further reservoirs in which *S. virchow* is maintained. The resulting collection of *S. virchow* isolates should be typed to determine any relatedness or clonality between the isolates. The results of this thesis recommend antibiotic susceptibility testing and phage typing be included as typing methods because of their proven discriminatory power. Ribotyping should also be included, despite its discriminatory power being lower than other methods because it did show accurate correlation with epidemiological data. It is anticipated that such an investigation would establish whether *S. virchow* is simply more widespread in Queensland than elsewhere in Australia, if *S. virchow* could be isolated more readily from sites in Queensland. Indications of other potential contributing factors may also be revealed, including any effect of climate by comparison of isolates from tropical regions of Australia with isolates from southern areas of Australia. Also factors such as

differences in population density or the proximity of intensive food animal production to urban areas may be able to be linked with prevalence of *S. virchow*.

The results of such a survey may also support a second hypothesis that the population of *S. virchow* in Queensland is epidemiologically distinct from other *S. virchow* in Australia. Perhaps the *S. virchow* in Queensland are members of an endemic clonal line that is particularly well adapted to its geographic location. If this was observed, investigation of the virulence factors of this clonal line would be significant. This could determine if the ability of some *S. virchow* isolates to cause invasive illness is due to enhanced expression of particular factors or the presence of a novel virulence factor.

The continued evaluation and application of typing methods is also an important objective in its own right. It is essential that the methods employed by epidemiologists to confirm the sources and cases in outbreaks are as accurate as possible. Therefore, it is imperative that the search for discriminatory and reliable methods to type strains be ongoing. PCR could lead the way in this area because it is capable of high volume throughput and offers sensitivity with the ability to detect very small numbers of organisms in a sample. Perhaps the focus for development in this area should be identifying optimal target regions for PCR, to allow sufficient homology to identify all cases but also enough diversity to distinguish outbreak strains from non-outbreak strains.

The approach taken in this thesis to elucidate the pathogenesis of *S. virchow* was to chose two determinants and study their potential roles in virulence. The SEF17 and LPS were chosen because they had been identified as candidate virulence factors in other serovars of *Salmonella* and were reasonably well studied in *Salmonella* (Palva & Mäkelä, 1980; Chart *et al.*, 1991b, 1993; Collinson *et al.*, 1991, 1993, 1996a; Doran *et al.*, 1993; Xiang *et al.*, 1993; Chart & Rowe, 1995). This approach was successful in demonstrating that SEF17 are expressed by *S. virchow* and warrant continued investigation to elucidate the control of expression and their mechanism of action in *S. virchow*. New variations in LPS structure with respect to the size of the core oligosaccharide and length of the O-side chain were also shown. In addition, the possibility of a novel pathway of LPS biosynthesis in *S. virchow* was

indicated by this study, which supports a finding by Chart *et al.* (1995). However, these are only small contributions in the larger context of understanding why *S. virchow* can cause extra-intestinal infections.

As most researchers would concede, the mechanisms and virulence factors involved in bacterial pathogenesis are numerous and it is likely that many are yet to be identified. One of the most important aspects of pathogenesis must surely be the interaction between an organism and the host cell. In prospect, studies which investigate the signals that occur both within the bacterium and between the bacterium and the host cell, have much to offer the understanding of how microorganisms causes disease. This type of approach would also allow elucidation of the possible regulation of bacterial virulence factors by the host cells or the environment. A molecular genetic strategy using reporter genes has been successfully applied to achieve this goal. This technique involves using a transposon that contains a promoterless marker gene such as an antibiotic resistance gene. If the transposon inserts downstream of an active promoter the antibiotic resistance is expressed. Bellofatto *et al.* (1984) used a transposon derivative in the study of *Campylobacter crescentus* and Finlay (1992) also reported using this approach to identify *S. typhimurium* genes which were induced when the bacterium binds to the host epithelial cell. Staendner *et al.* (1995) also employed this approach but utilized the chloramphenicol acetyltransferase (CAT) assay system, to identify promoters activated in *S. typhi,* during invasion into eucaryotic cells. Studies such as these involving *S. virchow* might elucidate whether its host factors, virulence of strains of *S. virchow* or a combination of these two factors that result in *S. virchow* being associated with both invasive and non-invasive illnesses.

In addition there are several other areas that are likely to be deserving of investigation in *S. virchow* that were not in the scope of this study. LPS variation was shown in some of the *S. virchow* isolates and this leads to the question, would these strains also have different levels of serum resistance? Serum resistance is a mechanism by which pathogens avoid a key host defence mechanism, and is achieved by preventing complement lysis of the bacterial cell. In some *Salmonella* species this prevention is due to the presence of O-antigens in the LPS molecule, which provide steric hindrance and inhibit access of the C5b-9 complex to the

bacterial surface (Finlay & Falkow, 1989a). In providing this hindrance the LPS protects the bacterial cell from lysis by complement. In light of the findings that different *S. virchow* isolates have different LPS and in particular different lengths of O-antigen, it is conceivable that they also have different serum resistance. Serum resistance could therefore be involved in the mechanism by which some strains survive to cause an invasive infection. The outer membrane protein, Rck, which is encoded on the large serotype-specific plasmid of *S. typhimurium* has also been shown to confer serum resistance on *S. typhimurium*, independently of LPS (Hackett *et al.*, 1987; Gulig, 1990; Cirillo *et al.*, 1996). Therefore the investigation of *S. virchow* for the presence of a high molecular weight serotype-specific virulence plasmid may also be valuable.

Another aspect of a bacterium's ability to avoid the host's defences is the ability to survive in macrophages. *Salmonella* replicates in the reticuloendothelial system, not the epithelial cells, as is the case for other enteric pathogens such as *Shigella* (Finlay & Falkow, 1989a). *S. typhimurium* has been shown to multiply within macrophages but there is a lag period of several hours before maximal intracellular division rates are reached (Finlay & Falkow, 1989b). Therefore assays such as the cell culture assay employed in this thesis should be used only to study adherence and invasion and macrophage cell lines should be used to study the potential for intracellular replication of *S. virchow*..

Lastly, invasion genes of *Salmonella* could also be studied in *S. virchow*. A great deal is known about the invasion locus of *Salmonella* including the presence of at least 28 genes arranged over ca. 35kb of the chromosome (Galán, 1996). Some of these genes have been shown to be necessary for entry into host cells and others are associated with a secretion system for proteins, which are involved in the signalling that leads to bacterial uptake by host cells. There are also regulatory genes that control the secretion process (Galán, 1996). This section of chromosome has been reported to be unstable in some serovars resulting in deletions and non-virulent strains (Galán, 1996). Therefore studies into the invasion locus in *S. virchow* might find deletions in some strains that are responsible for those strains being avirulent, while others are more invasive. A PCR assay could be developed which allows determination of whether the entire block of genes is present or if deletions have

occurred. Isolates could also be tested using the tissue culture invasion assay to establish whether strains with deletions in the invasion locus have less invasive ability than strains with the full invasion locus.

This thesis has provided many insights into the epidemiology and pathogenicity of *S. virchow*. The study of *S. virchow* still has many exciting findings to offer and much could be added to the knowledge of *S. virchow,* in particular its pathogenesis, by applying some of the approaches suggested in this discussion. Elucidation of the pathogenic mechanisms of *S. virchow* could be of great value, not only to the understanding of *S. virchow*, but of all *Salmonella*.

APPENDICES

Appendices

Appendix 1 - Antimicrobial agents and their properties

Penicillins

The penicillins were the first antibiotics used in therapy with the introduction of penicillin G in 1942 (Lancini et al., 1995). Penicillin is a member of the β-lactam class of antibiotics and *Penicillium* spp. are responsible for producing the naturally occurring penicillins (Balows & Hausler, 1991). More recently synthesis or alteration to the natural β-lactam structure has resulted in the production of the cephamycins, carbapenems, oxapenems and monobactams. The common mechanism of action by all of the β-lactam antibiotics is inhibition of bacterial cell wall synthesis. Resistance to the β-lactam antibiotics is by one of four described mechanisms; inactivation of the antibiotic by β-lactamases encoded either on the chromosome or extra-chromosomally, alteration of the target site, change in permeability of the target cell, or tolerance (Lancini et al., 1995).

Aminoglycosides

Aminoglycosides have been important in the treatment of Gram-negative infections since the introduction of streptomycin in 1944 (Balows & Hausler, 1991). The mode of action is to inhibit protein synthesis by binding to ribosomal subunits thus preventing translation and translocation during protein synthesis. Resistance can be due to more than one mechanism including enzymatic inactivation of the antibiotic, change in permeability of the target cell or modification of the site of action (Lancini et al., 1995). Gentamicin is relatively effective against members of the *Enterobacteriaceae* (Lambert & O'Grady, 1992).

Chloramphenicol

Chloramphenicol is another antibiotic with a fairly broad spectrum of activity. It acts by blocking transpeptidation by binding to the 50S ribosomal subunit. Chloramphenicol was once the drug of choice for the treatment of

salmonellosis (Lancini et al., 1995). In most cases of gastroenteritis caused by salmonellae the infection is self-limiting and recovery is not enhanced by the use of antibiotics. However, for infections such as typhoid or paratyphoid fever, caused by *S. typhi* and *S. paratyphi* A as well as infections in immunosuppresed patients, antibiotic treatment such as chloramphenicol was recommended (Simon et al., 1985). In more recent times chloramphenicol has been replaced by co-trimoxazole (one of the sulphonamide/trimethoprim antibiotics) as the drug of choice because the side effects are less severe (Simon et al., 1985).

Macrolides

Macrolides are named as such because they consist of a large lactone ring. There are two groups, the anti-bacterial and the anti-fungal macrolides. Erythromycin is one of the better known antibacterial macrolides in therapeutic use and it has activity against Gram-positive and some Gram-negative bacteria. The enteric bacteria such as *E. coli* and *Salmonella* are generally resistant to this antimicrobial agent.

Metronidazole

Metronidazole is a particularly effective antibiotic for the treatment of infections by anaerobic bacteria and protozoa. It is the best known member of the group of antibiotics derived from nitroimidazole. The bactericidal effect of metronidazole is due to the nitro group which once inside a susceptible cell is reduced, producing free radicals or cytotoxic metabolites. Resistance to this antibiotic is still fairly rare.

Nitrofurantoin

Nitrofurantoin is an example of a class of compounds (the furans) which have a nitro group joined to a heterocyclic ring. This antibiotic is most commonly used in the treatment of urinary tract infections and has a broad spectrum of activity (Balows & Hausler, 1991). The mechanism of action is not clear but at high concentrations it has been shown to cause inhibition of enzymes of the citric acid cycle and also

inhibit DNA, RNA and protein synthesis. It appears that clinical resistance to this antibiotic is rare (McOsker & Fitzpatrick, 1994).

Quinolones

Quinolones are a group of antibiotics related to naladixic acid. Many of the original quinolones have limited application because of widespread resistance. The new generation quinolones such as ciprofloxacin have been synthesized and are termed flouroquinolones because a flourine atom has been attached to the nucleus. The spectrum of activity of these antibiotics includes both Gram-negative and Gram-positive bacteria but they are not effective against anaerobic bacteria. Their mode of action is to inhibit DNA synthesis by inhibiting the enzyme DNA gyrase. Some resistance to these antibiotics occurs in the presence of acidic pH, divalent cations and urine (Balows & Hausler, 1991).

Sulphonamides

The sulphonamides are derived from sulphanilamide and have an antibacterial effect by competing with para-aminobenzoic acid for conversion to dihydrofolate, a step in the metabolism of folate. Sulphonamides show a synergistic effect when used in combination with trimethoprim. Trimethoprim also acts by interrupting folate metabolism but at a different site to sulphonamides. Ultimately bacterial DNA synthesis is prevented. The use of sulphonamide/trimethoprim is recommended in veterinary practice for the treatment of *Salmonella* infections (Cooper, 1994).

Tetracyclines

Tetracyclines have been in use clinically since the late 1940s and have a broad spectrum of activity against bacteria, both Gram-positive and Gram-negative, and also protozoa (Lancini et al., 1995). The mode of action is to inhibit protein synthesis by binding to the 30S ribosomal subunit. Tetracycline resistance is due to changes in the permeability of the target cell leading to a decrease in entry and an increase in the efflux of the antibiotic.

Appendix 2 - Polyacrylamide gel constituents

Polyacrylamide gels

Resolving Gel

12%		1 gel *
	distilled water (ca. 21°C)	10.05ml
	1.5M Tris-HCl, pH8.8 (ca. 4°C)	7.5ml
	10% (w/v) SDS (ca. 21°C)	0.3ml
	Acrylamide/Bis (30% stock) (ca. 4°C)	12.0ml
	–> DEGAS	
	10% ammonium persulfate (APS) (ca. 21°C)	150µl
	TEMED (ca. 21°C)	15µl

Stacking gel

4%		1 gel *
	distilled water (ca. 21°C)	3.05ml
	0.5M Tris-HCl, pH6.8 (ca. 4°C)	1.25ml
	10% (w/v) SDS (ca. 21°C)	50µl
	Acrylamide/Bis (30% stock) (ca. 4°C)	650µl
	–> DEGAS	
	10% ammonium persulfate (APS) (ca. 21°C)	25µl
	TEMED (ca. 21°C)	5µl

* These volumes are for the 20cm plates and 1mm spacers from the Protean II xi Vertical Electrophoresis cell (Bio Rad Laboratories, Inc.)

30% acrylamide/bis stock

mix 58.4g of powdered acrylamide and 1.6g of N'N'-Bis-methylene-acrylamide and make up to 200ml with distilled water. Store at 4°C and in a dark bottle

10% APS

dissolve 0.035g of powdered APS in 350µl of sterile distilled water. This must be prepared fresh daily

PAGE sample (solubilization) buffer

distilled water 50ml
0.5M Tris-HCl, pH6.8 ..... 12.5ml
glycerol 10ml
10% (w/v) SDS 20ml
2β-mercaptoethanol 5ml
0.05% (w/v) bromophenol blue 2.5ml

100ml

(store at room temperature)

10 x running (glycine) buffer

tris base 30g
glycine.....144g
SDS 10g
make up to 1L with distilled water and adjust to pH8.3
dilute 1:10 to use

Appendix 3 - Calculation of the numerical index of discrimination

The index of discrimination for each of the typing methods and the combination of all methods was calculated as outlined by Hunter and Gaston (1988).

The calculation of the index for plasmid profiling is provided as an example.

Plasmid profiling differentiated six types from a population of 104 isolates. The distribution of isolates was 15 in plasmid profile 1, 3 in PP2, 2 in PP3, 1 each in PPs 4 and 5 and 82 in PP 6. Therefore N=104, n_1=15, n_2=3, n_3=2, n_4=1, n_5=1, n_6=82 and

$$D = 1 - [(15 \times 14 + 3 \times 2 + 2 \times 1 + 1 \times 0 + 1 \times 0 + 82 \times 81)/(104 \times 103)]$$
$$= 1 - (6860/10712)$$
$$= 0.359$$

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